Functional Analysis of Different Streptococcal Invasion Mechanisms and Consequences for Intracellular Survival.

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von Katja Clara Branitzki-Heinemann
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1. Referent: apl. Prof. Dr. Manfred Rohde
2. Referent: Prof. Dr. Michael Steinert
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Go confidently in the direction of your dreams! Live the life you've imagined.

Thoreau

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LIST OF ABBREVIATIONS

°C	degree Celsius
%	percentage
APS	ammonium persulfate
ARF	acute rheumatic fever
AroD	aromatic amino acid rich domain
Arp2/3 complex	actin-related protein 2/3 omplex
BSA	bovine serum albumin
cfu	colony forming unit
dH_2O	deionized H_2O
DGE (Asp-Gly-Glu)	aspartic acid - glycine - glutamic acid
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EBM	endothelial basal medium
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
EGM	endothelial groth medium
EM	electron microscopy
ery^r	erythromycin resistent
EsB	energy selective backscattered electron
et al.	*et alii* (Latin 'and others')
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FCS	fetal calf serum
FESEM	field emission scanning microscopy
Fn	fibronectin
FnBR	fibronectin binding repeat
FnBP	fibronectin binding protein
GAS, GCS, GGS	group A, C, G streptococci

GEF	guanin nucleotide exchange factors
GfbA	group G fibronectin binding protein A
GfbApro	truncated GfbA derivate lacking the AroD region
g/l	gram per liter
GTPase	Guanosintriphosphatase
GST	Glutathion-S-Transferase
h	hour
HEP-2	Laryngeal epithelial cell line
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
i.e.	*id est* (Latin “that is”)
IFN	interferon
IF	imunofluorescence
IgG	immunoglobulin G
IL	interleukin
ILK	integrin-linked kinase
IPTG	Isopropyl-β-D-galactopyranoside
kan^r	kanamycin resistant
kV	kilo volt
LB	luria bertani
MBCD	Methyl-β-cyclodextrin
µg ml^{-1}	microgram per milliliter
MHC	major histocompatibility complex
min	minutes
µl	microliter
mM	millimol
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
NF	necrotizing fasciitis
PBS(T)	phosphate buffered saline (with Tween)
PCR	polymerase chain reaction
Pen/Strep	penicillin/streptomycin

PFA	paraform aldehyde
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethanesulfonylfluoride
ProD	proline rich domain
PSAGN	poststreptococcal acute glomerulonephritis
RGD (Arg-Gly-Asp)	arginine - glycine - aspartic acid
rpm	rounds per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SfbI	streptococcal fibronectin binding protein I
SfbIGaro	chimeric SfbI construct in which the AroD region was replaced by the homologue region of GfbA
SLO	streptolysin O
SLS	streptolysin S
SPE	streptococcal pyrogenic exotoxins
STSS	streptococcal toxic shock syndrome
TAE	Tris-acetate-EDTA
TEM	transmission electron microscopy
TEMED	N,N,N,N-Tetramethylethylendiamine
TGF	transforming growth factor
THB	Todd Hewitt Broth
THY	Todd Hewitt Yeast
TNF	tumor necrosis factor
TSB	Tryptic Soy Broth
U	unit
V	volt
v/v	volume/volume
w/v	weight/volume
WB	western blot
α	alpha
β	beta
γ	gamma

1. ABSTRACT

S. pyogenes subvert the host immune defense mechanisms by several different strategies including invasion of host cells. SfbI, the major adhesion and invasion factor consists of a domain structure with a N-terminal aromatic amino acid rich domain (AroD), a central proline rich domain (ProD) and a C-terminal Fn-binding region. Central and C-terminal regions consist of repeats (ProD repeats, Fn-binding repeats (FnBRs)) which are sequence conserved but differ highly in their number within SfbI proteins of different clinical isolates. While the FnBRs and their interaction with fibronectin are well characterized no information was available for the function of the AroD and of the ProD regions. Bound Fn bridges SfbI to host cell integrins; SfbI triggers integrin-clustering and caveolae-aggregation on the cellular surface subsequently resulting in bacterial internalization within membrane invaginations. SfbI-expressing streptococci avoid a classical intracellular endosomal-lysosomal trafficking route which would lead to phagolysosomal killing of the pathogen.

The AroD and the ProD region might affect SfbI-mediated uptake processes since it was observed that, due to the different modular structure of SfbI, besides the already described entry process a morphological different internalization mechanism showing typical features of classical phagocytosis such as the formation of membrane protrusions was initiated. Analysis of co-incubation assays from recombinant protein-constructs, latex beads coated with recombinant protein or *Streptococcus gordonii* heterologous expressing distinct SfbI-constructs with host cells revealed that distinct AroD types or lack of the ProD avoid typical SfbI-internalization events. Integrin-clustering and caveolae-aggregation were prevented; rather cytoskeleton rearrangements and signaling events of classical phagocytic processes were induced. Moreover, the intracellular trafficking route has consequences for bacterial survival, since after uptake via cytoskeleton rearrangements intracellular streptococci co-opt the classical endosomal-lysosomal pathway leading to successful bacterial killing, whereas bacterial survival is benefitted by bypassing phagolysosomal fusion caveolae-based entry. In addition ProD is supposed to modify host cellular integrin-composition with own distinct integrin-binding sides and furthermore prevent actin cytoskeleton remodeling by directly inhibiting Arp2/3 complex dependent actin-branching affecting internalization processes and intracellular trafficking.

2. INTRODUCTION

Members of the genus *Streptococcus* (from Greek *kokkos*: spherical; *streptos*: chain) are catalase-negative, non-motile Gram-positive oval or spherical bacteria which grow in pairs or chains. Streptococci are metabolically anaerobes since they do not use oxygen even under normoxic growth conditions; they belong to the homofermentative bacteria producing lactic acid from glucose metabolism. Streptococci require enriched media for optimal culturing since they are nutritionally fastidious, thus bacterial colonies are usually small and streptococci are not found free-living in the environment. Among relatively avirulent normal microbial flora organisms of animals and humans also some pathogens with a broad significance in medicine and industry belong to this heterogeneous group of bacteria (Hardie, 1986; Patterson, 1996; Ruoff and Bisno, 2010; Moschioni *et al*., 2010).

Members of the medically important streptococci species are *S. pyogenes* the main cause of *e.g.* pharyngitis, scarlet fever and late immunologic sequelae such as glomerulonephritis (for details see 2.1.1), *S. pneumonia* the classical cause of community acquired pneumonia, *S. agalactiae* the common agent of neonatal disease and the oral streptococci including *S. mutans* and *S. sanguis* which were shown to be involved in dental caries and *S. mitis* which was found to be associated with bacteremia and periodontal disease (Patterson, 1996; Ruoff and Bisno, 2010; Moschioni *et al*., 2010). Various streptococci were furthermore studied for their ability to induce zoonoses in domestic and wild animals (Timoney, 2004; Agnew and Barnes, 2007; Lun *et al*., 2007).

The nomenclature of *Streptococcus* species is highly complex and designations have been largely based on haemolysis type or serological grouping rather than species names (Patterson, 1996; Moschioni *et al*., 2010). In 1903 Schottmüller made a first attempt to classify the genus *Streptococcus* based on their hemolytic properties on blood-agar (Schottmüller, 1903). Up to today haemolysis typing is an important aspect for classification of streptococci (Facklam, 2002). A greenish discoloration on blood-agar is dependent on the oxidization of iron in hemoglobin molecules within red blood cells (α-hemolytic species), whereas β-hemolytic streptococci cause complete rupture of red blood cells appearing as areas clear of blood in the vicinity of bacterial colonies on blood agar. γ-hemolytic species have no hemolytic capacities (Brown, 1919; Figure 1).

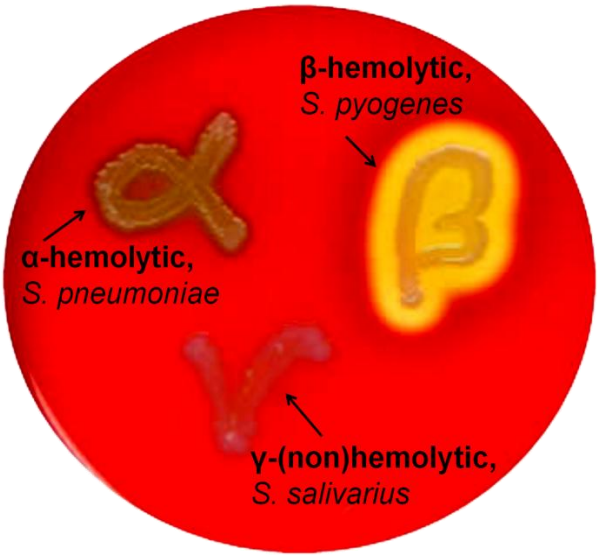

Figure 1: Hemolytic properties of streptococci (modified from http://drugline.org/img/term/hemolysis-6959_1.jpg)

The ß-hemolytic organisms were taxonomically further subdivided into group A-V streptococci on the basis of group specific carbohydrate or lipoteichoic acid antigens, a scheme developed by Rebecca Lancefield (Lancefield, 1933).

During the last decades it became apparent that one of the most useful tools for classifying genetic relatedness among *Streptococcus* strains is phylogenetic typing based on 16S rRNA gene sequencing. However, the phenotypic characteristics mentioned above are still useful for the putative identification of many commonly encountered streptococci species (Facklam, 2002; Ruoff and Bisno, 2010; Moschioni *et al*., 2010).

The expression of a characteristic membrane bound virulence factor which was shown to trigger virulence of *Streptococcus (S.) pyogenes* is the M protein which is used for further subdividing *S. pyogenes* into serotypes. By *emm* typing more than 130 distinct M genotypes could be identified in the last decades (Beall *et al*., 1996; Steer *et al*., 2009[a], Smeesters *et al*., 2010).

2.1 *Streptococcus pyogenes*

S. pyogenes is also known as β-hemolytic group A streptococcus (GAS) and is the most pathogenic bacterium within the *Streptococcus* genus (Facklam, 2002). This exclusively human pathogen with high medical impact is responsible for a broad range of human diseases including not only local infections of the respiratory tract and the skin (*e.g.* pharyngitis and pyoderma) but also life-threatening invasive infections, like streptococcal toxic shock syndrome (STSS), bacterimia and necrotizing fasciitis. Although, in the majority of cases infections proceed relatively harmless, GAS cause more than 500,000 deaths

annually worldwide and severe delayed, nonsuppurative sequelae, such as acute glomerulonephritis and acute rheumatic fever, may follow uncomplicated streptococcal infections (Cunningham, 2000; Bisno *et al*., 2003; Carapetis *et al*, 2005). GAS subverts the various host immune defense mechanisms by different strategies including the expression of numerous secreted and non-secreted virulence factors (Cunningham, 2000; Courtney *et al.*, 2002; Nizet, 2007; Nobbs *et al*., 2009). Even though traditionally viewed as extracellular pathogens, GAS is known for the potential to adhere to and invade into host cells (LaPenta *et al*., 1994; Greco *et al*., 1995; Molinari *et al*., 1997; 2000; Jadoun *et al*., 1997; Cue *et al*., 1998; Dombeck *et al*., 1999; Cywes and Wessels, 2001). Indeed, *S. pyogenes* is still sensitive against the antibiotic penicillin, but internalization into host cells might prevent complete bacterial killing and therefore allows the streptococci to persist within the host, leading to spreading and colonizing of deeper tissue (Österlund *et al.*, 1997; Neeman *et al*., 1998; Kreikemeyer *et al*., 2004). The interactions with host cells to initiate invasion are mediated by surface and secreted virulence factors and often contain interaction with extracellular matrix (ECM) molecules especially fibronectin. The major fibronectin-binding protein of GAS is SfbI (streptococcal fibronectin binding protein I) and its allelic variant, protein F1, which plays a key role in bacterial adherence and host cell invasion (Hanski and Caparon, 1992; Talay *et al*., 1992; 2000; Molinari *et al*., 1997; Ozeri *et al*., 1998).

2.1.1 GAS pathogenesis

One of the most frequently found human pathogens worldwide is GAS which is responsible for more than 700 million cases of pharyngitis and pyoderma annually, and which causes more than 650,000 invasive infections like necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (Carapetis *et al*., 2005; Olsen *et al*., 2009, Nizet and Arnold, 2012). Postinfectious immune sequelae like acute rheumatic fever (ARF) or poststreptococcal acute glomerulonephritis (PSAGN) are furthermore dreaded especially in medically underserved populations where overcrowding and poor access to health care are contributing factors (Olsen and Musser, 2010). Nonsuppurative sequelae thought to result from autoimmune responses based on streptococcal molecular mimicry and are contributed by specific characteristics of the pathogens, susceptibility of the host, and environmental factors (Cunningham, 2000; Nizet and Arnold, 2012). The major long-term complications of GAS infections are ARF experienced subsequently to pharyngitis and PSAGN which in some cases follows skin infections like impetigo (Courtney *et al*., 2002; Nizet and Arnold, 2012).

Antigenic similarity between molecular constituents of GAS, i.e. superantigens, and human tissue result in immunological crossreactions and might account for the clinical manifestations of ARF and PSAGN (Bisno *et al*., 2003) More than 15 million people worldwide with rheumatic hard disease (RHD) the most serious complication of ARF have to face long-term morbitiy and mortility rates (Tani, 2008; Nizet and Arnold, 2012); 233,000 deaths annually can be attributed to ARF or RHD (Carapetis *et al*., 2005).

The human oropharynx is the most frequent GAS infection site but also smallest skin lesions can provide entry ports for GAS (Tart *et al*., 2007; Olsen and Musser, 2010; Nizet and Arnold 2012). School-age children 5 to 15 years of age are mainly affected by GAS infections i.e. pharyngitis and scarlet fever (Cunningham, 2000; Bisno *et al*., 1995) but many individuals carry GAS asymptomatically in the upper respiratory tract and other anatomic sites like the skin (Tart *et al*., 2007); in general individuals of all ages can get infected with GAS when not possessing type-specific immunity (Nizet and Arnold, 2012). Asymptomatic carriage or outbreak of the disease might be a challenge of numerous factors: mutations in the pathogen which regulate virulence, or a (in) productive immune response by the host that affects pathogen proliferation, or the internalization of the pathogen into host cells influencing antibiotic treatment success and GAS spreading might influence the infection outcome (Tart *et al*., 2007; Olsen *et al.*, 2009; Nizet and Arnold, 2012). Moreover, individuals infected with the same strain can develop very different infectious manifestations; that made the important role of host environment, co-existing disease and genetics become evident (Olsen *et al*., 2009). Spreading of the pathogen is realized by direct person-to-person contact with mucus from the nose or throat of infected individuals or with infected wounds on the skin; thus, spreading is especially facilitated under crowded conditions, such as those at schools (Cunningham, 2000; Olsen and Musser, 2010; CDC, 2013).

In the U.S.A. and Western Europe severe invasive GAS infections had become uncommon during the second half of the 20th century (Katz and Morens, 1992; Nizet and Arnold, 2012), however, the rate has re-emerged during the last decades. Each year more than 3 per 100,000 people in industrial societies and up to 80 per 100,000 people of the indigenous population in North Queensland, Australia are infected with severe invasive GAS disease; the infections are associated with a high mortality rate with worldwide 160,000 deaths annually (Stevens, 1996; O'Loughlin *et al*., 2007; Tart et al., 2007; Lamagni *et al.,* 2008; Olsen and Musser, 2010). The GAS "flesh-eating disease" NF involves the skin, subcutaneous and deep soft tissue, and muscle of the patient and is characterized by extensive tissue damage, vascular dissemination and systemic disease manifestations. Invasive severity might occur in a matter of hours to days by progression of a previous superficial bacterial infection such as pharyngitis or from an apparently benign appearing

skin lesion (Olsen *et al*., 2009; Olsen and Musser, 2010, Nizet and Arnold, 2012). As GAS proliferates in normally sterile environment the host immune system responds with a rapid influx of acute inflammatory cells. The release of tissue-damaging enzymes by immune cells such as neutrophils combined with degradative virulence factors expressed by invading GAS subsequently provoke necrosis of adjacent tissues (Olsen and Musser, 2010). STSS is characterized by hypotension, shock and multiple organ failure and may be accompanied by NF.

Different risk factors were found to enhance susceptibility for severe GAS infections. For example varicella infection significantly increased the risk for acquisition of invasive GAS disease in previously healthy children (Laupland *et al*., 2000); diabetes mellitus, HIV infections, intravenous drug abuse, chronic pulmonary or cardiac disease, or alcoholism are risk factors causing a relative immunosuppression and thus, predisposing adults to severe invasive GAS infections (Olsen *et al.,* 2009; Olsen and Musser, 2010; Nizet and Arnold, 2012).

Since no effective vaccine to prevent GAS infections is yet available a better understanding of GAS virulence is mandatory (Olsen *et al.*, 2009). Vaccination would clearly constitute an alternative strategy for control of GAS infection (Fritzer *et al.,* 2012) but vaccine development is challenging especially because of the high diversity of conserved surface-associated GAS protein antigens, large variations in the geographical distribution and the production of antibodies cross-reactive with human tissue which can lead to host auto-immune disease like ARF (Fritzer *et al*., 2010; Nizet and Arnold, 2012). Vaccination would not only reduce the burden of GAS disease but also the treatment with antibiotics and thus might prevent the development of resistance in group A streptococci (Fritzer *et al.,* 2010). Several streptococcal target antigens have been investigated as potential vaccine candidates including cell membrane associated and secreted anchorless proteins like Fn-binding proteins or pilus components (Guzmán *et al*., 1999; Bisno *et al*., 2003; Steer *et al*., 2009[b]; Henningham *et al*., 2012) but the best studied vaccine target is the GAS M protein. However, the number and the diversity of M proteins and the risks of molecular mimicry and autoimmune disease hinder M protein-based vaccine development (Fae *et al*., 2006; Martins *et al*., 2008; Fritzer *et al*., 2010).

2.1.2 Virulence factors

The key steps for initiation of an infection are (i) adhesion of the bacteria to host cells for example via Fn-binding proteins. (ii) adjustment of the pathogen to the surrounding milieu i.e. to pressure, hypoxia, temperature and of course to host immune defense mechanisms by changing the expression pattern of genes benefitting bacterial adaption such as invasins for internalization of host cells or pore-forming toxin for resistance to phagocytic clearance. (iii) subversion of the host innate immunity for example by expressing capsular structures or by interfering with host complement function.

GAS express strain dependent distinct virulence factors based on their genetic endowment and their growth phase and dependent on the infected tissue side with consequences on the local environmental factors (Bisno *et al*., 2003). The rapid respond to local changes leads to establishment and dissemination of the infection (Tart *et al*., 2007). The numerous secreted and surface-bound virulence factors allow *S. pyogenes* to challenge the naturally outstanding host immune defense strategies resulting in an excessive wide spectrum of GAS disease (Tart *et al*, 2007; Nizet, 2007).

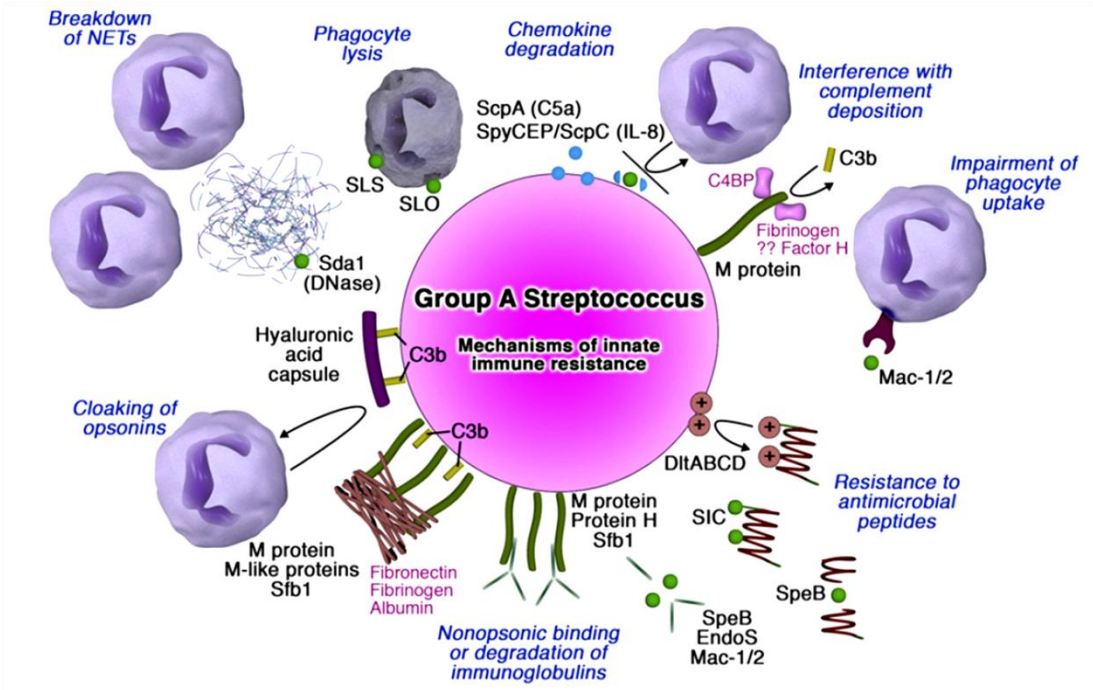

Figure 2: Mechanisms by which GAS subverts host innate immune defense (from Nizet, 2007).

Secreted virulence factors include the streptococcal pyrogenic exotoxins (SPE) which are a family of more than 15 secreted factors with the capacity to act as bacterial superantigens. GAS expresses several superantigens such as SPEs, exotoxin F or streptococcal superantigen (SSA) which simultaneously bind MHC class 2 molecules and the T-cell receptor (Marrack and Kappler, 1990; Bisno *et al.*, 2003). The resulting antigen-independent activation of large numbers of T lymphocytes promotes the release of TNF-α, IL-1β, IL-2; IL-6 and IFN-γ subsequently causing widespread organ dysfunction, disseminated intravascular thrombosis and tissue injury (symptoms occurring in GAS depending diseases such as ARF or STSS) (Hauser *et al*., 1991; Stevens, 1995; Cunningham, 2000; Nizet and Arnold, 2012).

Further secreted key virulence factors of GAS are streptolysin O (SLO) and streptolysin S (SLS). Both pore-forming hemolysins are produced by almost all GAS strains, are toxic to various host cell types including macrophages and neutrophils and damage the membranes of subcellular organelles leading to impaired phagocytic capacity (Nobbs *et al*., 2009; Olsen *et al*., 2009; Nizet and Arnold, 2012). SLO is oxygen-labile and features the property to enhance intracellular GAS-survival within epithelial cells by preventing lysosomal internalization of the bacteria which might benefit long-term carriage. SLS is oxygen-stabile and responsible for the β-hemolytic nature of GAS on blood agar (Bisno *et al.*, 2003; Hakansson *et al*., 2005; Nizet, 2007; Nobbs *et al*., 2009; Olsen *et al*., 2009; Nizet and Arnold, 2012).

Streptokinase, another secreted factor, is a plasminogen-activating protein produced by all GAS isolates that promotes plasmin-accumulation on the bacterial surface by catalyzing the conversion of plasminogen to plasmin. Streptococci prevent encapsulation with host fibrin which is hydrolyzed by surface associated plasmin and therefore spreading of GAS through tissues and development of invasive infections is advanced (Sun *et al*., 2004; Nobbs *et al*., 2009).

Streptococci are encapsulated pathogens. The capsule is composed of hyaluronic acid and is chemically similar to human connective tissue resulting in molecular mimicry of host antigens and minimized recognition of GAS by the immune system (Patterson, 1996; Bisno *et al.*, 2003). Furthermore, the hyaluronate capsule was shown to prevent opsonized phagocytosis by neutrophils or macrophages, to mediate streptococcal adhesion to CD44-positive host cells, to benefit resistance against bactericidal activities of different toxic substances such as radicals or bacteriocins and to promote GAS cathelicidin antimicrobial peptides resistance and survival within neutrophil extracellular traps (Courtney *et al*., 2002; Bisno *et al.*, 2003; Cole *et al*., 2010).

The GAS serotyping classification system is based on M protein a major GAS surface associated virulence factor which contributes to GAS virulence in multifarious ways (Olsen

and Musser 2010; Ghosh, 2011). M protein was shown to be involved in streptococcal adhesion to and invasion into host cells (Okada *et al*., 1995; Cue *et al.*, 1998) and in neutralization of antimicrobial peptides (Nilsson *et al.*, 2008; Lauth *et al*., 2009). It prevents phagocytotic processes impeding complement-based neutrophil recognition (Bisno *et al*, 2003) and precluding the fusion of azurophilic granules with phagosomes (Staali *et al.*, 2006). At the same time M proteins contain antigenic epitopes mimicking those of mammalian muscle and connective tissue and therefore leading to autoimmune sequelae with the subsequent onset of an acute GAS infection since crossreactive antibodies target those bacterial and host antigenic structures (Gosh, 2011). However, not all M-types exhibit the same structures or functions and therefore differ in their effect during GAS infections (Courtney *et al*., 2002).

Furthermore, GAS strains express several surface associated proteins which were shown to bind human fibronectin resulting in adherence to and internalization of the pathogen into host cells (for details see 2.3). The fibronectin-binding proteins (FnBPs) belong to the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family of adhesins and are known to play an important role in streptococcal pathogenesis since they allow the streptococci to hide from antibiotics and host immune mechanisms (Schwarz-Linek *et al*., 2004; 2006). The most prominent of these proteins is SfbI (or its allelic variant PrtF1) which also binds IgG, thus, preventing antibody-dependent cell cytotoxicity by macrophages (Medina *et al*., 1999). Importantly, FnBPs are significantly more prevalent among persisting GAS strains recovered from asymptomatic carriers (Tart *et al*., 2007), thus playing a key role in recurrent and not successfully treatable infections by occupying safe ecological niches within the host.

2.2 *Streptococcus dysgalactiae* subspecies *equisimilis*

Streptococcus (S.) dysgalactiae subspecies *equisimilis* (SDSE) is a β-hemolytic streptococcus isolated from human and animals and closely related to GAS (Facklam, 2002). Most of the veterinary pathogens could be distinguished from those of human origin by extensive taxonomic studies during the past years; however, the genetic relationship between animal and human isolates remains controversial (Brandt and Spellerberg, 2009). SDSE strains of the Lancefield type A, C, G and L were identified in human infections but the group antigens C and G are the most frequently found (group C and G streptococcus; GCS

and GGS) (Brandt *et al.*, 1999; Facklam, 2002; Brandt and Spellerberg, 2009). SDSE was long been considered as nonpathogenic but has now recognized as important human pathogen. SDSE is known for causing various human infections including pharyngitis, meningitis, endocarditis and sepsis. Furthermore, also severe invasive diseases like necrotizing soft tissue infections can be produced and GCS and GGS could be associated with ARF (Brandt *et al*., 1999; Humar *et al.*, 2002; Davis *et al*., 2007). Since GCS and GGS are usually not identified to the species level exact quantitative information about SDSE infections are not available; nonetheless the isolation frequency is increasing and it is reported that invasive SDSE infections approximated that of invasive GAS infections (Brandt *et al*., 1999; Davis *et al.*, 2007; Broyles *et al*., 2009). In human SDSE colonizes the skin, the throat, the female genital and the gastrointestinal tracts (Brandt *et al.*, 1999; Brandt and Spellerberg, 2009). SDSE expresses various virulence factors which show high similarity to those from GAS such as SPEs, SLS/ SLO, capsule, M protein and FnBP (Brandt and Spellerberg, 2009; see Table 1).

GCS/ GGS share not only phylogenetic relationships with GAS but also colonize a similar ecological niche, express homologous virulence factors and thus not surprising, exhibit considerable disease profiles (Davis *et al.*, 2007).

2.2.1 Pathogenesis

Missing information about infection number hardly permits rating the impact of SDSE infections. It is well accepted that the streptococci are transmitted from person to person without any animal origin. Zoonotic GCS and GGS infections are rare; most cases of zoonotic infections are due to other streptococci species (Brandt and Spellerberg, 2009). SDSE foremost affects immunocompromised patients debilitated by old age or by suffering from multiple underlying diseases, predominantly malignancies but also injection drug users and burn patients are shown to be susceptible to SDSE infections (Brandt *et al.*, 1999; Brandt and Spellerberg, 2009). SDSE was identified as cause of various human infections with a comparable spectrum of GAS diseases ranging from relatively harmless to life-threatening dimensions. GCS and GGS were found responsible for superficial skin and soft-tissue infections such as cellulitis or pyoderma but also for wound infections and necrotizing fasciitis. SDSE can cause classical pharyngitis predominantly in adult patients, however, invasive infections comprising STSS, meningitis, endocarditis, and bacteremia were also

associated with GCS and GGS. Furthermore, reports on PSAGN and on ARF support the impact of SDSE in human infections (Turner *et al*., 1997; Brandt et al., 1999; Haidan *et al*., 2000; Barnham and Weightman, 2004; Broyles *et al*., 2009; Brandt and Spellerberg, 2009).

2.2.2 Virulence factors

SDSE expresses various virulence factors nearly identical to those from GAS like SPEs, SLS/ SLO, capsule, M protein and FnBP (Brandt and Spellerberg, 2009; see Table 1). SLS, for instance, provokes not only the β-hemolytic phenotype it also contributes to NF pathogenesis of SDSE (Humar *et al*., 2002).

Table 1: Virulence factors from GAS and SDSE (modified from Brandt and Spellerberg, 2009)

	Genes or homologues described in	
Proteins with highly similar sequences	SDSE	GAS
Fibronectin binding proteins	X	X
Plasmin(ogen) binding proteins	X	X
Glyceraldehyde-3-phosphate dehydrogenase	X	X
Streptococcal surface enolase	X	X
Protein S (vitronectin) binding protein	X	X
Laminin binding protein	X	X
Streptolysin O	X	X
Streptolysin S	X	X
Superantigens (SpeA, Spec, SpeG, SpeM, Ssa, and Smez)	X	X
Dysgalacticin	X	…
M protein	X	X
Capsule	X	X
C5a peptidase	X	X
Protein G	X	…
Streptokinase	X	X

Adhesins like M protein or FnBP, toxins like SLS or SLO and dissemination factors like capsule or streptokinase are putative virulence determinants of GGS and GCS described with molecular studies (Ikebe *et al.*, 2004). Horizontal gene-transfer events among streptococcal species of the pyogenic group are hypothesized to provide a basis for related virulence mechanism and similar spectrum of disease of SDSE compared to GAS (Brandt and Spellerberg, 2009).

Further research work needs to be done since many details about virulence factors and virulence mechanism of GCS and GGS are still not investigated. The capsule of GAS was extensively studied, whereas the role of the capsule for the pathogenicity of SDSE could just be speculated on. Genes corresponding to the *emm* gene of GAS are also present in SDSE but no relationship to the potential for invasiveness could be found and an inhibitory effect on phagocytosis as shown for GAS M protein remains to be elucidated (Schnitzler *et al*., 1995; Brandt and Spellerberg, 2009). The virulence factors may differ in their expression level compared to other SDSE strains but also to GAS strains making their effect on pathogenesis only presumable. Nonetheless, their existence is revealed and without any doubt SDSE significantly cause various human diseases.

2.3 Fibronectin binding proteins

The first essential step in initialization an infection involves attachment to and colonization of host cells (Kline *et al*., 1996; Henderson *et al.*, 2011; Yamaguchi *et al*., 2012). Internalization into host cells and intracellular persistence might be beneficial for bacterial survival by protecting the pathogen from antibiotic treatment or recognition and combating of the host immune system. Adhesion and invasion processes of bacteria are initialized by virulence factors often targeting fibronectin (Fn) a glycoprotein of the extracellular matrix. Fn circulating as a dimeric molecule in body fluids is assembled by cells into the fibrillar matrix via $\alpha_5\beta_1$ integrin on the cellular surface recognizing the RGD (Arg-Gly-Asp)-motif within the Fn molecule (Singh *et al.*, 2010). More than 100 FnBPs of Gram Positive and Negative bacteria have already been identified and especially streptococci strains express numerous FnBPs (Henderson *et al.*, 2011).

GAS is known for the potential to adhere to and invade into host cells (LaPenta *et al*., 1994; Greco *et al*., 1995; Molinari *et al*., 1997; 2000; Jadoun *et al*., 1997; Cue *et al*., 1998; Dombeck *et al*., 1999; Cywes and Wessels, 2001) and 11 FnBPs affecting host cell

association have been currently identified (Yamaguchi *et al*., 2012; Table 2). Some are prevalent in GAS isolates while others are expressed only by specific GAS serotypes (Natanson *et al*., 1995), nonetheless 97 % of clinical isolates of GAS strains express at least one FnBP. The existence of several FnBPs in GAS might be associable with the ability of streptococci to colonize different host tissues (Schwarz-Linek *et al*., 2006).

Table 2: Reported Fn-binding proteins of *S. pyogenes* (from Yamaguchi *et al.*, 2012)

	Full name	FnBRs	Characteristics
PrtF1/SfbI	Protein F1/*S. pyogenes* Fn-binding protein I	+	PrtF1/SfbI is composed of a signal sequence, aromatic domain, proline-rich region, functional upstream domain, Fn-binding repeats and a cell wall anchoring motif.
PrtF2/PFBP	Protein F2/*S. pyogenes* Fn-binding protein	+	PrtF2/PFBP contain two Fn-binding domains in the C-terminal regions, one of which consists of three Fn-binding repeats and the other is a non-repeated domain.
SOF/SfbII	Serum opacity factor/*S. pyogenes* Fn-binding protein II	+	SOF/SfbII is composed of an N-terminal opacification domain and C-terminal Fn-binding repeats region, and binds fibrinogen and fibulin-1.
SfbX	*S. pyogenes* Fn-binding protein X	+	SfbX is encoded immediately downstream of SfbII/SOF and contains Fn-binding repeats on the C-terminus.
Fbp54	Fbp54	+	Fbp54 is calculated to have a molecular mass of 54 kDa, and has a high similarity to Fn-binding proteins in other streptococci.
FbaA	Fn-binding protein of group A streptococci type A	+	FbaA contains a signal sequence, α-helical variable region, repeat domains and a cell wall anchoring motif. The *fbaA* gene is positively controlled by the *mga* gene.
FbaB	Fn-binding protein of group A streptococci type B	+	FbaB contains a signal sequence, Fn-binding repeats and a cell wall anchoring motif. The *fbaB* gene is regulated by the *msmR* gene, as well as the *prtF1/sfbI* and *prtF2/pfbp* genes.
M1 protein	M1 protein	−	M1 protein is composed of two polypeptide chains that form an α-helical coiled coil configuration, while the chains are built of four repeat regions and a cell wall anchoring motif.
Shr	Streptococcal haemoprotein receptor	−	Shr contains two 'near transporter' domains that mediate Fn and laminin binding.
Scl1	Streptococcal collagen-like surface protein 1	−	Scl-1 has been reported to be streptococcal collagen-like protein and contains a variable region, collagen-like region and cell wall anchoring domain.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	−	GAPDH protein, located in the cytoplasm and on the bacterial cell surface, plays important roles in the glycolytic pathway and is essential for bacterial growth.

The GAS FnBPs SfbI/PrtF1, PrtF2/PFBP, FbaA, FbaB, SfbII/ SOF, SfbX and Fbp54 possess C-terminal Fn-binding domains containing different numbers of Fn-binding repeats (FnBR) which realize the interaction with Fn (Schwarz-Linek *et al*., 2006).

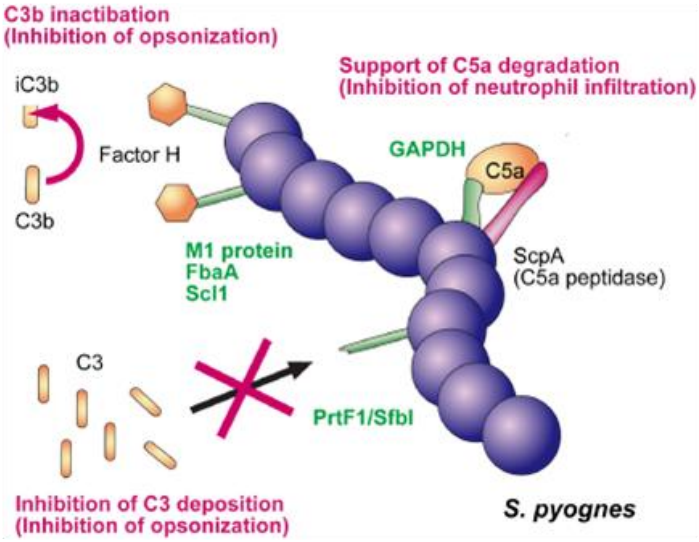

Figure 3: Schematic diagram for the evasion of host innate immunity through FnBPs (from Yamaguchi *et al*., 2012).

Some FnBPs of GAS, i.e. M1 protein, SfbI/PrtF1, FbaA, Scl1 and GAPDH, are furthermore involved in other virulence mechanisms like evasion of innate immunity by inhibiting complement activity (Yamaguchi *et al.*, 2012; Figure 3).

FnBPs trigger streptococcal internalization into host cells and thus occupy safe ecological niches within the host. They are found significantly more prevalent among persisting GAS strains which were recovered from asymptomatic carriers and might play an important role in recurrent and not successfully treatable GAS infections by residing within host cells (Tart *et al*., 2007).

2.3.1 SfbI and GfbA

The major Fn-binding protein of GAS is SfbI (streptococcal Fn-binding protein I) and its allelic variant, protein F1, which is, moreover, the best-characterized adhesin of GAS and plays a key role in bacterial adherence and host cell invasion (Hanski and Caparon, 1992; Talay *et al*., 1992; 2000; Molinari *et al*., 1997; Ozeri *et al*., 1998). As a member of the classical MSCRAMM family it consists of a N-terminal signal sequence mediating secretion across the bacterial membrane, a central effector region and a C-terminal membrane-spanning region with an LPXTG motif that is recognized by the sortase enzyme and covalently attached to cell wall peptidoglycan (Henderson *et al*., 2010). The effector region consists of a modular organized conserved domain structure (Talay *et al.*, 1994; Towers *et al*., 2003; Figure 4): co-operative Fn-binding is implemented by the C-terminal FnBRs and the upstream spacer

region. The function for the central proline rich domain (ProD), consisting of repeats, could not be elucidated yet, but for the N-terminal aromatic amino acid rich domain (AroD) binding of fibrinogen and a modulatory effect on the invasion process of streptococci was shown recently (Katerov *et al*., 1998; Rohde *et al*., 2011).

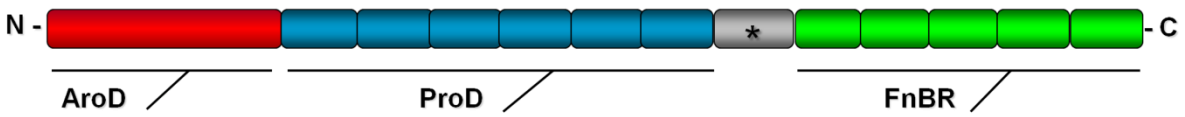

Figure 4: Schematic illustration of the domain structure of the SfbI protein (*= spacer**).**

In 2003 Towers *et al*. analyzed the *sfbI* genes of numerous clinical GAS isolates and showed how variable the domain structure is distributed among different clincial *S. pyogenes* strains. Various types of the AroD region were identified and it was observed that SfbI proteins are highly variable in their number of ProD repeats and FnBRs in a strain dependent manner. A possible effect of different number of repeats on *e.g.* Fn-binding and consequences for adherence and invasion processes have not been investigated so far. Although a single SfbI protein can bind several Fn-dimers it is not finally clarified if there need to be a critical density of Fn inducing intergrin clustering and subsequent receptor signaling leading subsequently to streptococcal uptake (Schwarz-Linek *et al*., 2003; 2006).

In 1996 Kline and colleagues identified a SfbI homologue in GGS, termed GfbA for group G streptococcal fibronectin-binding protein A, and observed GfbA-mediated adherence to host cells. GfbA proteins contain identical domain structure and show significant amino acid identity with SfbI proteins (more than 75 %); the number of ProD repeats and FnBRs of SfbI and GfbA are highly similar, whereas the AroD regions differ considerably (Kline *et al*., 1996). Interspecies gene transfer between GAS and GGS is hypothesized to form the basis for the homologous virulence factors SfbI and GfbA. Both factors are expressed under the transcriptional regulation of RofA within the chromosomal FCT (fibronectin, collagen binding, and T antigen) locus which suggests analogous regulatory mechanisms of protein expression and would allow homologous recombination between the streptococcal (Towers *et al*., 2004).

2.4 Bacterial invasion into non-professional phagocytic host cells

Several pathogens are capable of inducing their own entry into non-professional phagocytic host cells. In order to escape immune defense processes they can trigger their internalization by subverting the cellular actin cytoskeleton. Activation of the Rho family GTPases Rac1 and Cdc42 is common mechanism to stimulate cytoskeleton rearrangements during internalization; for example *Salmonella typhimurium* uses a type III secretion system to translocate virulence factors into host cells where they directly affect the GTPases, whereas *Listeria monocytogenes* and *Neisseria sp.* induce receptor-dependent cellular signaling cascades leading to Rac1 activation, and subsequent engagement of downstream mediators (Rottner *et al*., 2005; Figure 5).

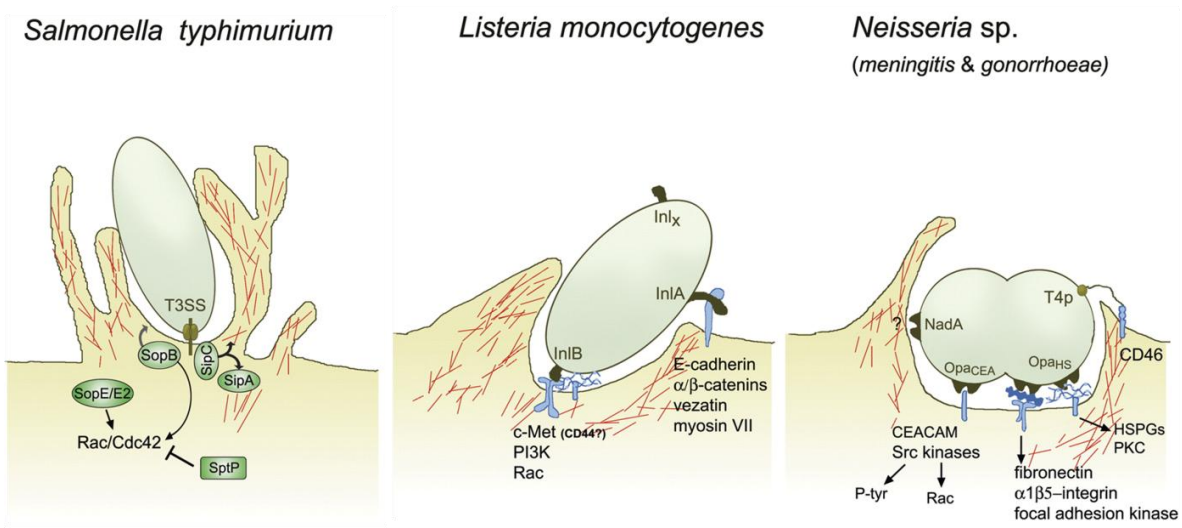

Figure 5: Schematic illustrations of the cytoskeleton rearrangements in bacterial internalization (modified from Rottner *et al*., 2005).

Also for GAS internalization processes the involvement of GTPases is described; Ozeri and colleagues investigated the recruitment and activation of Rac and a possible participation of Cdc42 during SfbI-mediated entry of GAS (Ozeri *et al*., 2001). Both GTPases were also shown to be activated during cell adhesion and spreading on the ECM protein Fn in an integrin-mediated manner (Price *et al*., 1998). Integrins play a key role in bacterial entry processes; *Neisseria*, enteropathogenic *Yersinia* as well as *Staphylococcus aureus* and GAS mediate adhesion to and invasion into host cells by exploiting integrin signaling (Ozeri *et al*., 1998; Massey *et al.*, 2001; Wong and Isberg, 2005; Rottner *et al*., 2005).

The internalization of GAS depends on Fn acting as a bridging molecule to $\alpha_5\beta_1$-integrins on the host cell surface (Molinari *et al*., 1997; Ozeri *et al*., 1998). Different morphological pattern were reported based on the FnBP modulating the entry process: M1 protein was shown to induce streptococcal uptake via cytoskeleton rearrangements whereas SfbI triggers the aggregation of caveolae resulting in the formation of large invaginations and the subsequent uptake of streptococci (Dombek *et al*., 1999; Molinari *et al*., 2000; Rohde *et al.*, 2003).

Caveolae are omega-shaped invaginations of the plasma membrane of several mammalian cells such as epithelial and endothelial cells and are suggested to function as signaling centers involved in endocytic processes (Anderson, 1998). Several pathogens including bacteria such as *Mycobacterium kansaii*, *Chlamydia trachomatis* and FimH-expressing *Escherichia coli*, viruses or parasites but also SfbI expressing GAS were reported to induce caveolae-dependent entry into host cells (Peyron *et al*. 2000; Norkin *et al*., 2001; Shin and Abraham, 2001[a]; 2001[b]; 2001[c]; Majomäki *et al.*, 2002; Rohde *et al*., 2003). SfbI triggers the aggregation of caveolae with subsequent fusion on the host cell surface resulting in the uptake of streptococci via large invaginations (Molinari *et al*., Rohde *et al*., 2003).

Fn is bound to FnBPs of GAS inducing in a conformational change in the glycoprotein which finally provides access of the $\alpha_5\beta_1$-integrin recognition RGD-motif. The exposed motif can now interact with cell surface bound integrins and trigger bacterial internalization (Courtney and Podbielski, 2004; Yamaguchi *et al*., 2012). Integrin engagement leads to phosphoinositide 3-kinase (PI3K) dependent activation of integrin-linked kinase (ILK) subsequently promoting paxilin phosphorylation. Focal adhesion kinases (FAK) finally link integrin signaling to the actin cytoskeleton inducing large cytoskeleton rearrangements in the case of M1 protein (Molinari *et al*., 2000). SfbI-triggered streptococcal uptake furthermore promotes Src-induced phosphorylation of caveolin-1 which up-regulates lipid raft dependent caveolae-endocytosis (Wang *et al*., 2006[b]; 2007; Yamaguchi *et al.*, 2012; see Figure 6).

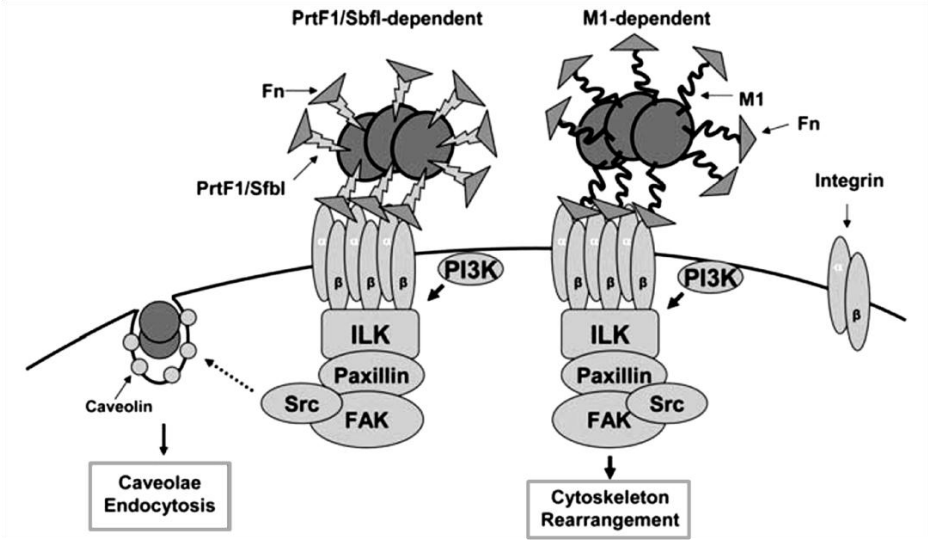

Figure 6: Schematic diagram of postulated signaling pathways that are mediated by M1 and Sfbl proteins leading to group A streptococcal entry (modified from Wang et al., 2007).

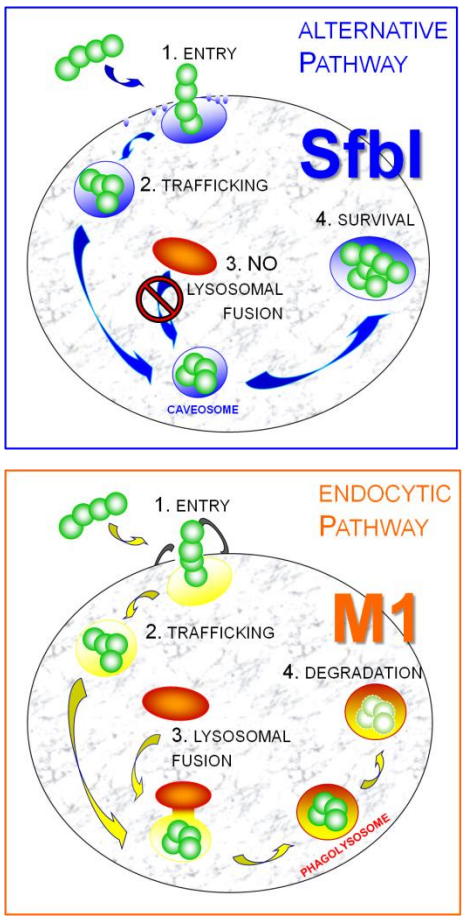

Figure 7: Schematic illustrations of the intracellular trafficking route dependent on the internalization process with hypothetical outcome.

Intracellular trafficking inside the host cells can differ significantly; some reside within phagosomes and modify the compartmental milieu as it was shown for *Salmonella*, whereas others escape and survive within the cytosol like *Shigella* (Rottner *et al*., 2005). It is not yet clarified which strategy is implemented by GAS trafficking intracellular along the classical endosomal route with subsequent lysosomal fusion after entry via cytoskeleton rearrangements, however, after caveolae-dependent internalization streptococci were shown to traffic and reside intracellular within caveosomes. By co-opting the caveolae-mediated intracellular trafficking route SfbI-expressing GAS avoid fusion with lysosomes and thus prevent exposure to the killing machinery (Molinari *et al*., 2000; Rohde *et al*., 2003; for details see Figure 7).

It is hardly believable that the same initialization via the interaction of Fn with $\alpha_5\beta_1$-integrins and similar signaling cascades result in the observed morphological different internalization processes triggered by M1 and SfbI proteins.

Wang and colleagues hypothesized an effect from Fn-binding characteristics of M1 and SfbI since both streptococcal virulence factors bind different Fn domains with distinct affinities (Wang *et al*., 2007). Furthermore, it was shown that M1 additionally mediates Fn-independent streptococcal uptake by binding CD46 (Rezcallah *et al*., 2005) and Ozeri and colleagues demonstrated that next to $\alpha_5\beta_1$-integrins also $\alpha_V\beta_3$- integrins play a role in SfbI-dependent entry processes (Ozeri *et al.*, 1998). The involvement of distinct receptors may have serious consequences for cellular signal events required for different invasion pattern (Yamaguchi *et al*., 2012).

Since the FnBRs of SfbI can hypothetical bind numerous Fn-dimers (Schwarz-Linek *et al*., 2004) it is not surprising that Rohde and coworkers observed significant SfbI-induced integrin-clustering on the host cell surface visualized by the aggregation of gold nanoparticles coated with recombinant protein (Rohde *et al.*, 2003); there might be differences in receptor-clustering in M1 or SfbI-triggered internalization changing the signaling events inside the host cell. Indeed, an enhanced uptake of GAS initialized by SfbI was reported when $\alpha_5\beta_1$-integrins were upregulated by TGFβ_1 a cytokine that increases the α_5 integrin subunit expression and which production is promoted by GAS (Wang *et al*., 2006[a]).

Additionally, Fn has been shown to bind various other host factors including collagen, gelatin, DNA, fibrin, fibulin and interact with a wide variety of integrins (Pankov and Yamada, 2002; Henderson *et al.*, 2010). Dinkla *et al*. (2003) recently demonstrated collagen recruitment via SfbI-bound Fn with consequences for aggregation, colonization and immune evasion of GAS. Interaction of FnBPs with a specific Fn domain, distinct conformational changes and the number of bound Fn-dimers might further affect the binding-capacity of Fn-molecules to those other factors mentioned above, and thus potentially influencing subsequent processes.

3. AIM OF THE STUDY

Fibronectin-binding proteins seem to play a significant role in streptococcal persistence by affecting bacterial recurrence and ineffective antibiotic treatment. It is essential to observe mechanisms underlying streptococcal virulence such as internalization into host cells to receive a better understanding of this highly important human pathogen and to offer continuative treatment strategies against GAS infections in the future.

The first essential step in initialization of a bacterial infection involves attachment to and colonization of host cells (Kline *et al*., 1996; Henderson *et al.*, 2011; Yamaguchi *et al*., 2012). Internalization and persistence might benefit bacterial survival and infection outcome by protecting the pathogen from antibiotic treatment or recognition and combating of the host immune system. Adhesion and invasion processes of group A and G streptococci are triggered by an interaction with host cell integrins via the ECM molecule fibronectin. Binding to fibronectin is realized by the nearly identical Fn-binding proteins SfbI (GAS) (Talay *et al.*, 2000) and GfbA (GGS) (Kline *et al*., 1996) which exhibit a similar modular structure consisting of C-terminal Fibronectin-binding repeats, a central proline rich domain with different number of repeats and a N-terminal aromatic amino acid rich domain. SfbI and GfbA differ significantly only in their AroD region (Towers *et al*., 2004).

SfbI is known for inducing bacterial uptake by aggregation and subsequent fusion of caveolae and the formation of large membrane invaginations (Rohde *et al*., 2003). Preliminary electron microscopical analysis by Rohde and his colleagues revealed different entry mechanisms of GfbA expressing GGS inducing cytoskeleton rearrangements, and of SfbI expressing GAS, triggering caveolae-dependent uptake (Rohde *et al*., 2011). The authors constructed a truncated GfbA derivate lacking the AroD region (GfbApro) and generated a chimeric SfbI construct in which the AroD region was replaced by the homologue region of GfbA (SfbIGaro) to study the function of the AroD region in more detail.

The four proteins were heterologous expressed on the surface of the naturally non invasive SGO and were found to induce bacterial entry in different ways. The AroD region of GfbA triggered cytoskeleton rearrangements, independent of the GfbA or SfbI background, whereas the truncated GfbA induce a caveolae-dependent streptococcal uptake comparable

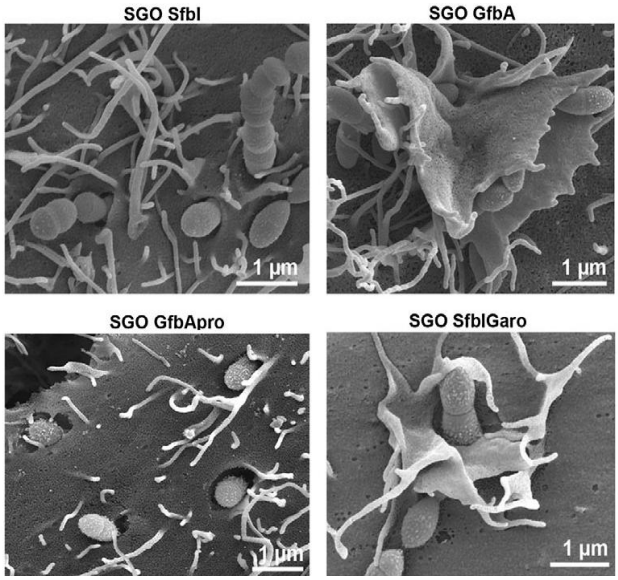

Figure 8: Internalization mechanisms induced by SGO heterologous expressing SfbI or GfbA constructs (from Rohde *et al*., 2011).

to the SfbI-induced entry mechanism. As already shown for SfbI mediated streptococcal invasion (Rohde *et al*., 2003) bacteria reside within caveosomes and prevent lysosomal fusion when the entry into host cells was mediated by the aggregation and subsequent fusion of caveolae which was found to be induced by SfbI and GfbApro. In contrast GfbA and SfbIGaro trigger bacterial uptake via large cytoskeleton rearrangements followed by trafficking intracellular along the classical endocytic pathway with final lysosomal fusion leading to the degradation of the streptococci.

In the present study differences in integrin-clustering induced by those recombinant proteins on the surface of endothelial host cells and visualized by the aggregation of gold-nanoparticles should be investigated with high resolution field emission scanning electron microscopy technique. Moreover, intracellular surviving *Streptococcus gordonii* heterologous expressing the distinct SfbI/ GfbA constructs should be enumerated to receive evidence for a correlation of the intracellular trafficking pathway, which depends on the bacteria entry mechanisms, and the effect on bacterial survival rates.

The role of the ProD region within SfbI proteins is completely unknown. Preliminary experiments of host cells infected with different clinical GAS isolates demonstrated distinct invasion mechanisms dependent on the SfbI-structure, for examples the number of ProD repeats and FnBRs. To study a possible function of the ProD in the SfbI-modulated internalization process SfbI constructs should be generated by inverse PCR technique consisting of different numbers of ProD repeats but identical N- and C-terminal backbone for not altering the function of the AroD and the FnBRs. Host cells should be co-incubated with latex beads coated with recombinant proteins to gain information about entry processes, involved host cellular structures, host cell signaling cascades and intracellular trafficking. The *sfbI* gene of GAS strain A40 which was used in the studies of Rohde *et al*. should serve as template for PCR approaches but since no gene sequence data were available first of all the gene needed to be sequenced.

4. EXPERIMENTAL PROCEDURES

4.1 Materials

Chemicals and media were purchased from following companies if not otherwise stated: BBinternational, Becton Dickinson, Calbiochem, Enzo, Fluka, Invitrogen, Merck, PAA, Promocell, Roth, Serva, Sigma.

Plastic disposables were obtained from the companies Greiner, Nunc and TPP.

4.2 Bacteria used in this study

In the present study bacteria listed below were used for the following specified experimental approaches.

Table 3: *S. pyogenes* strains used for infection assays

Strain	ProD repeat	FnBR	Reference / Origin
***S. pyogenes* A40**	6	4	Clinical pharyngeal isolate, Braunschweig, Germany
***S. pyogenes* A157**	3	3	Clinical skin isolate, Towers *et al*., 2003 (NS1042)*
***S. pyogenes* A171**	3	2	Clinical skin isolate, Towers *et al*., 2003 (NS297)*
***S. pyogenes* A184**	5	5	Clinical skin isolate, Towers *et al*., 2003 (NS539)*
***S. pyogenes* A310**	6	2	Clinical skin isolate, Towers *et al*., 2003 (NS495)*
***S. pyogenes* A316**	3	3	Clinical skin isolate, Towers *et al*., 2003 (NS1045)*

* Different nomenclatures result in different number of repeats compared to Towers *et al*., 2003.

Table 4: *E. coli* strains used for protein biochemical experiments

Strain	Plasmid	Insert	Reference
***E. coli* DH5α**	-	-	Strain collection of the department MMIK, HZI
***E. coli* DH5α**	pGEX-6p-1	-	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbI wt*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔ1ProD repeat*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔ2 ProD repeat*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔ3 ProD repeat*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔ4 ProD repeat*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔ5 ProD repeat*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔProD*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔFnBR*	present study
***E. coli* DH5α**	pGEX-6p-1	*sfbIΔFnBRΔAroD*	present study

Table 5: *S. gordonii* strains used for infection assays and survival studies

Strain	Specificity	Reference / Origin
SGO GfbA	SGO GP1221 background, GfbA from GGS G59, ery^r	R. Graham
SGO GfbApro	SGO GP1221 background, GfbA from GGS G59, ery^r	R. Graham
SGO SfbI	SGO GP204 background, SfbI from GAS DSM2071, kan^r	K. Dinkla
SGO SfbIGaro	SGO GP1221 background, SfbI from GAS DSM2071, GfbA from GGS G59, ery^r	R. Graham

4.3 Antibodies used in this study

Table 6: Antibodies

Antibody	Dilution	Company / Reference
Anti-Phalloidin Alexa Fluor® 568	1:200 (IF)	Invitrogen
Goat-anti-rabbit IgG Alexa Fluor® 488	1:200 (IF)/ 1:400 (FACS)	Invitrogen
Goat-anti-mouse IgG Alexa Fluor® 488	1:200 (IF)	Invitrogen
Mouse-anti-Arp2/3C1B, polyclonal, mouse	1:50 (IF)	Novus Biologicals
Rabbit-anti-human fibronectin IgG HRP-conjugated	1:1000 (WB)	Dako
Rabbit-anti-SfbI, polyclonal	1:75 (IF, FACS)	S. Talay

IF, Immune Fluorescence; WB, Western Blot

4.4 Microbiology

4.4.1 Media, solutions and instruments

Media were prepared with dH_2O and steam sterilized by autoclaving for 20 min at 121 °C before usage.

Table 7: Media for bacterial growth

LURIA BERTANI (LB)	Bacto tryptone	10 g/l
	NaCl	10 g/l
	Bacto yeast extract	5 g/l
TODD HEWITT YEAST (THY)	THB media powder	10 g/l
	Bacto yeast extract	5 g/l

TRYPTIC SOY BROTH (TSB)	TSB media powder	30 g/l

For preparation of agar-plates 15 g/l agar were added before autoclaving.

Table 8: Antibiotics for bacterial growth

	Supplier	Application for culturing	Final concentration
AMPICILLIN	Applichem	*E. coli* expressing pGEX-6P-1 derivates	100 µg ml^{-1}
ERYTHROMYCIN	Sigma	SGO GfbA, SGO GfbApro, SGO SfbIGaro	3 µg ml^{-1}
KANAMYCIN	Roth	SGO SfbI	500 µg ml^{-1}

Table 9: Solutions and instruments used for microbiological experiments

	Company
CO_2 incubator	Thermo Scientific
Glycerol	Roth
Incubator shaker	Infors
37 °C incubator	Haereus

4.4.2 Growth conditions and culturing

GAS were routinely grown in TSB media at 37 °C over night without agitation, SGO were grown respectively in the presence of antibiotics as specified in Table 8. For survival studies SGO were plated on THY-agar with relevant antibiotics and incubated over night at 37 °C.

E. coli strains were cultured in LB supplemented with ampicillin (Table 8) over night at 37 °C and shaking (120 rpm).

For long-term storage 750 µl bacteria of an overnight culture were mixed with 250 µl sterile glycerol and stored at - 80 °C.

4.5 Molecular biological procedures

4.5.1 Materials (i.e. buffer, kits, chemicals) and instruments

Buffers were prepared with dH_2O and steam sterilized by autoclaving at 121 °C for 20 min before usage.

Table 10: Buffers and media for molecular biological experiments

SOB MEDIA	Bacto tryptone	2 % (w/v)
	Bacto yeast extract	0.5 % (w/v)
Sterilized by autoclaving for 20 min,	NaCl	10 mM
at 1 bar high pressure and 121 °C.	KCl	2.5 mM
TFBI MEDIA	Potassium acetate	30 mM
	RbCl	100 mM
Adjusted to pH 5.8,	$CaCl_2$	10 mM
sterile filtrated and stored at 4 °C.	$MnCl_2$	50 mM
	Glycerol	15 % (v/v)
TFBII MEDA	MOPS	10 mM
	$CaCl_2$	74 mM
Adjusted to pH 6.5,	RbCl	10 mM
sterile filtrated and stored at 4 °C.	Glycerol	1.5 % (v/v)
TRIS/ACETATE/EDTA (TAE)	Tris-HCl (pH 7,4)	50 mM
	NaCl	150 mM
	EDTA	1 mM

Table 11: Solutions, kits and instruments for molecular biological experiments

Kits	**Company**
DNeasy Blood & Tissue Kit	Qiagen
PCRExtract Mini Kit	5Prime
QIAprep Spin Miniprep Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Solutions	
dNTPs [2 mM]	Fermentas
ethidium bromide	Roth
GeneRuler DNA Ladder Mix	Fermentas
Phusion High-Fidelity DNA Polymerase [2 U/µl]	Thermo Scientific
Restriction enzymes & provided buffers	JenaBioscence
Taq DNA Polymerase [5 U/µl]	Qiagen
5 x HF-buffer	Thermo Scientific
6 x loading dye	Fermentas
Instruments	
Centrifuge 5804 R	Eppendorf
Centrifuge 5417 R	Eppendorf
EASY-System K429	Herolab
electrophoresis chamber and power source	Biorad
Thermocycler	Biometra
UV-Transilluminator	Herolab UVT 2020

4.5.2 Isolation of chromosomal DNA from GAS and plasmid DNA from *E. coli*

Chromosomal DNA from *S. pyogenes* strain A40 served as template for experiments resulting in the cloning of the whole and the truncated *sfbI*-genes (sfbI *wt*, *sfbI* Δ*FnBR*, *sfbI* Δ*FnBR*Δ*AroD*) into the pGEX-6p-1 vector. Chromosomal DNA was isolated using the DNeasy Blood & Tissue Kit following the supplementary protocol.

Plasmid DNA (i.e. pGEX-6p-1 including SfbI derivates) from *E. coli* was isolated with the QIAprep Spin Miniprep Kit following manufacturer's advices.

DNA was eluted in the provided elution buffer or in MQ H_2O and stored at - 20 °C. DNA integrity was observed by agarose gel electrophoresis (for detailed information see 4.5.3).

4.5.3 Agarose gel electrophoresis

This technique is used for separation and analysis of DNA molecules within an electric field, i.e. after successful plasmid isolation or for assessing insert sizes after restriction digest or PCR. Separation of DNA molecules was carried out by gels made from 1 % (w/v) agarose in TAE buffer; samples were mixed with 6 x loading dye and separated by 120 V for 25 - 45 min. GeneRuler DNA Ladder Mix was used as molecular weight standard. Before DNA fragments were visualized with an UV-Transilluminator and documented with an EASY-System the agarose gel was incubated with 1 % ethidium bromide.

4.5.4 Polymerase Chain Reaction (PCR), inverse PCR and colony PCR

PCR-method amplifies DNA fragments of defined nucleotide sequence and length.

Experiments were performed using the oligonucleotides specified in Table 12 in a Thermocycler according to the standard protocol with.

The *sfbI wt* gene of GAS A40 and the truncated derivates *sfbI* Δ*FnBR* and *sfbI* Δ*FnBR*Δ*AroD* lacking the signal sequence (SS) and the membrane anchor (MA) were amplified by standard PCR and used for cloning approaches in the pGEX-6P-1 vector system (Figure 9).

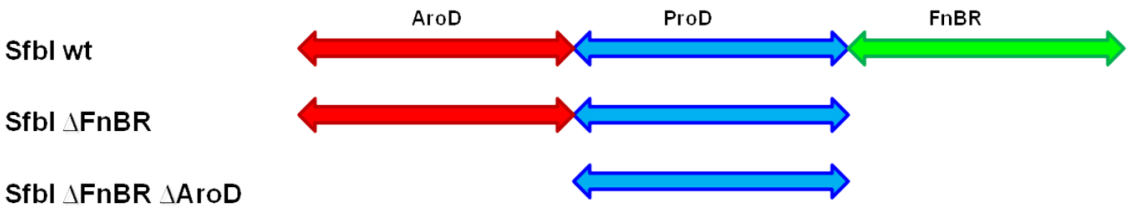

Figure 9: Schematic overview of the SfbI, SfbI ΔFnBR and SfbI ΔFnBRΔAroD derivates

The plasmid *pGEX-sfbI wt* served as template for the inverse PCR experiments, in which *sfbI*-constructs were generated, differing in the number of ProD repeats. Following constructs were generated with the specified primer: ***sfbI Δ1*** (fwd1_SfbI_iPCR and rev_SfbI_iPCR), ***sfbI Δ2/ sfbI Δ3/ sfbI Δ4*** and ***sfbI Δ5*** (fwd2_SfbI_iPCR and rev_SfbI_iPCR), ***sfbI ΔProD*** (fwd6_SfbI_iPCR and rev_SfbI_iPCR). The whole plasmid was amplified; only the fragment omitted by the primer pairs was excluded. The linear amplification products were later-on restriction digested and relegated (for details see Figure 10).

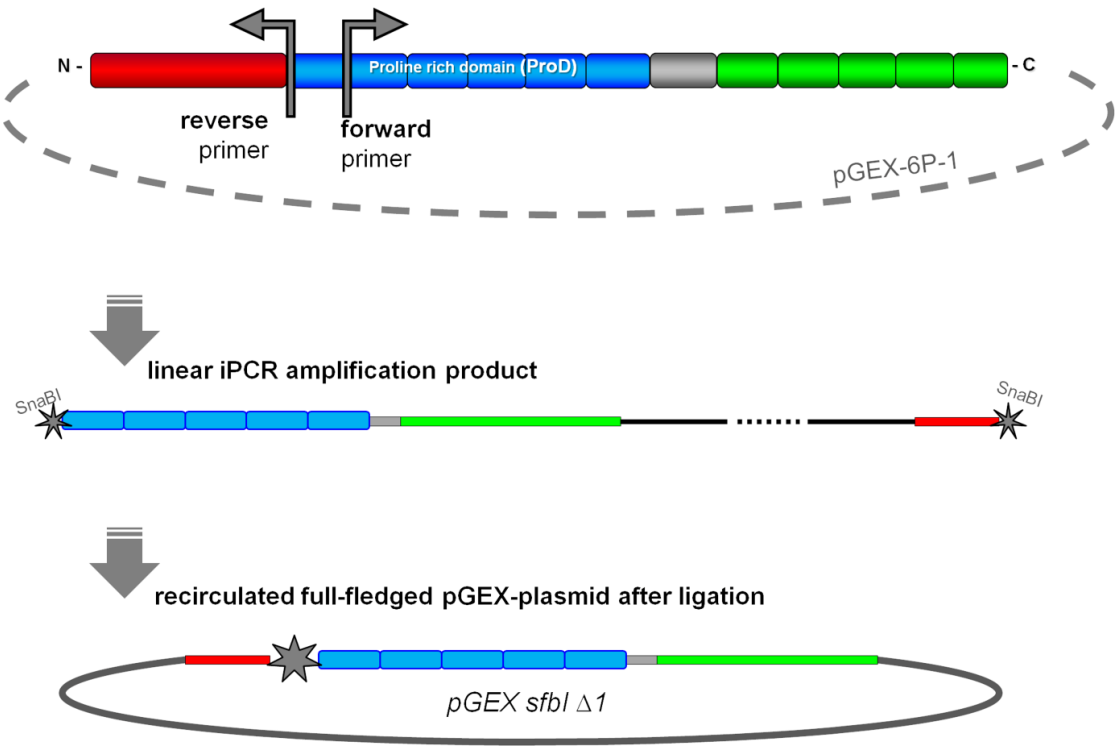

Figure 10: Schematic overview of the iPCR principle using as example the generation of *sfbI Δ1*

Using the colony PCR method the existence of a specific insert sequence within a single *E. coli* colony after transformation was investigated. As template a single colony was resuspended in 13 µl sterile deionizated milli Q (MQ) H_2O and heated for 4 min in a microwave.

Table 12: Oligonucleotides

Name	**Sequence 5' – 3'**	**Restriction enzyme**
PCR for amplification of *sfbI* (lacking SS and MA)		
BAMHI_4ER (FORWARD)	GGGGGATCCGCGGATGAGAAGACTGTGCCG	BamHI
SALI_234,310,20 (REVERSE)	CCCGTCGACGATAGGTGTTATATTTTTTGTAGG	SalI
PCR for amplification of *sfbI* ∆*FnBR* (lacking SS and MA)		
BAMHI_4ER (FORWARD)	GGGGGATCCGCGGATGAGAAGACTGTGCCG	BamHI
PRR_SALI (REVERSE)	CCCGTCGACCAATAGGTAAGTCAACACT	SalI
PCR for amplification of *sfbI* ∆*FnBR*∆*AroD* (lacking SS and MA)		
PRR_BAMHI (FORWARD)	GGGGGATCCCTTTTAAGTGCTGAATATGTACC	BamHI
PRR_SALI (REVERSE)	CCCGTCGACCAATAGGTAAGTCAACACT	SalI

Inverse PCR		
FWD1_SFBI_IPCR (FORWARD)	GGGTACGTACCCGAGGACCCAAAACGTC	SnaBI
FWD2_SFBI_IPCR (FORWARD)	GGGTACGTACCAGAAGTTCCAAGCGAGAGC	SnaBI
FWD6_SFBI_IPCR (FORWARD)	GGGTACGTACCTGAAAAACCTAGTGTTGAC	SnaBI
REV_SFBI_IPCR (REVERSE)	CCCTACGTATACATATTCAGCACTTAAAAG	SnaBI
Colony PCR		
PGEX5' (FORWARD)	GGGCTGGCAAGCCACGTTTGGTG	-
PGEX3' (REVERSE)	CCGGGAGCTGCATGTGTCAGAGG	-
Sequencing		
PCR12 (FORWARD)	GCGGGTACCCAATATTTTCTCAAAAAATCA	KpnI
PRRR (REVERSE)	CAATAGGTAAGTCAACACT	-
PRRF (FORWARD)	AATCTTTTAAGTGCTGAATATGTACC	-
PCR13 (REVERSE)	GCGTCTAGATTATCCACTATTCAGCATATTTGC	XbaI

SS, signal sequence; MA, membrane anchor; underlined are the restriction sides

Table 13: PCR protocols

PCR approach		PCR program		
PCR for amplification of *sfbI wt* (1), *sfbI* ∆*FnBR* (2) and *sfbI* ∆*FnBR*∆*AroD* (3)				
0.5 µl	TEMPLATE DNA [~ 0.15 µg/µl]	98 °C	2 min	DENATURATION
10 µl	5 X HF-BUFFER	98 °C	0.5 min	DENATURATION
2 µl	FORWARD PRIMER [10 pmol/µl]	x 25 60 °C	1 min	ANNEALING
2 µl	REVERSE PRIMER [10 pmol/µl]	72 °C	x min*	ELONGATION
5 µl	DNTP STOCK [2 mM]	72 °C	5 min	FINAL
0.5 µl	PHUSION HIGH-FIDELITY DNA POLYMERASE [2 U/µL]	4 °C	∞	POLYMERISATION
30 µl	MQ H_2O			
		* APPLICATION-DEPENDENT ADJUSTED: 2.5 MIN (1), 1.5 MIN (2) OR 1 MIN (3)		
Inverse PCR				
0.3 µl	TEMPLATE DNA	98 °C	0.5 min	DENATURATION
10 µl	5 X HF-BUFFER	98 °C	0.1 min	DENATURATION
1.5 µl	FORWARD PRIMER [10 pmol/µl]	x 25 x °C*	0.5 min	ANNEALING
1.5µl	REVERSE PRIMER [10 pmol/µl]	72 °C	x min**	ELONGATION
5 µl	DNTP STOCK [2 mM]	72 °C	5 min	FINAL
0.5 µl	PHUSION HIGH-FIDELITY DNA POLYMERASE [2 U/µL]	4 °C	∞	POLYMERISATION
31.0 µl	MQ H_2O			
		* /**APPLICATION-DEPENDENT ADJUSTED: * 60 °C, 50 °C, 60 °C/ ** 1.5 MIN - 4 MIN		
Colony PCR				
13 µl	TEMPLATE (MQ H_2O WITH COLONY MATERIAL, BRIEFLY BOILED)	96 °C	5 min	DENATURATION
10 µl	10 X BUFFER	96 °C	1 min	DENATURATION
1 µl	FORWARD PRIMER [10 pmol/µl]	x 29 59 °C	1 min	ANNEALING
1 µl	REVERSE PRIMER [10 pmol/µl]	72 °C	2 min	ELONGATION
2 µl	DNTP STOCK [2 mM]	72 °C	5 min	FINAL
2 µl	$MgCl_2$ [25 mM]	4 °C	∞	POLYMERISATION
0.3 µl	TAQ DNA POLYMERASE [5 U/µL]			

4.5.5 Restriction digest and ligation

PCR amplification product (*sfbI wt, sfbI ΔFnBR and sfbI ΔFnBRΔAroD*) and pGEX-6P-1 vector were restriction digested with the enzymes BamHI and SalI over night at 37 °C before purification and ligation (for details see Table 14).

iPCR amplification products were restriction digested with SnaBI for 4 h at 37 °C before purified and recirculated by ligation (for details see Table 14).

Table 14: Transformation protocols

Restriction digest		**Ligation experiment**	
for *sfbI wt*, *sfbI ΔFnBR* and *sfbI ΔFnBRΔAroD* with pGEX-6P-1			
30 µl	ELUATED AMPLIFICATION PRODUCT OR VECTOR	2 µl	VECTOR
		10 µl	INSERT
4 µl	10 X BUFFER (B4)	2 µl	10 X T4-LIGASE BUFFER
1 µl	BAMHI [10 U]	0.5 µl	T4-DNA LIGASE [5 U]
1 µl	SALI [10U]	5.5 µl	MQ H_2O
4 µl	MQ H_2O		
Inverse PCR			
50 µl	ELUATED AMPLIFICATION PRODUCT	20 µl	SAMPLE
5,75 µl	10 X SNABI-BUFFER	2.5 µl	10 X T4-LIGASE BUFFER
1.5 µl	SNABI [15U]	0.3 µl	T4-DNA LIGASE [5 U]

Ligation samples were stored at - 20 °C and directly used for transformation experiments in *E. coli* DH5α.

4.5.6 Transformation of *E. coli*

Preparation of competent bacteria

E. coli was grown over night at 37 °C with agitation (120 rpm) in SOB media. Overnight culture was diluted 1:100 in fresh SOB media and further incubated until bacteria reached early exponential growth phase ($OD_{578\ nm}$= 0.4 - 0.6). Culture was cooled down on ice for 15 min before bacteria were collected by centrifugation at 5000 rpm for 10 min at 4 °C and resuspended in 15 ml TfbI. After another centrifugation step bacteria were resuspended in 4 ml TfbII and incubated on ice for 15 min. Competent bacteria were aliquoted, immediately shocked-frozen in liquid N_2 and stored at - 80 °C.

Transformation of competent bacteria

10 µl ligation approaches were carefully mixed with 50 µl competent *E. coli* and incubated for 30 min on ice to allow adsorption of DNA to bacterial membrane. After 90 sec heat shock at 42°C which temporarily looses the membrane 50 µl pre-warmed LB media was added and the culture was incubated at 37 °C with shaking (120 rpm) for at least 1 h. Bacteria were selected on LB-agar in the presence of ampicillin.

Via colony PCR clones with a positive insert signal were inoculated in LB media; overnight cultures were used for glycerol stock cultures (see 4.4.2) and for plasmid DNA preparation (see 4.5.2). All potential constructs were verified by nucleotide sequence analysis before continuative experiments.

4.6 Protein biochemical procedure

4.6.1 Materials (i.e. buffer, kits, chemicals) and instruments

Buffers were prepared with dH_2O and were steam sterilized by autoclaving at 121 °C for 20 min before usage.

Table 15: Buffers for protein biochemical approaches

COOMASSIE-STAINING SOLUTION	0.1 % (w/v)	Coomassie Brilliant Blue
	40 % (w/v)	Methanol
	10 % (w/v)	Acetic acid
ELUTION BUFFER	10 mM	Glutathione
	50 mM	Tris (pH 8.0)
PBS (PH 7,4)	1.44 g/l	Na_2HPO_4
	0.24 g/l	KH_2PO_4
	8 g/l	NaCl
	0.2 g/l	KCl
PBST		PBS
	0.05 % (v/v)	Tween-20
RESOLVING GEL	1.9 ml	dH2O
	1.7 ml	Acryamide [30 %]
	1.3 ml	Tris-HCl [1.5 M] (pH 8.8)
	50 µl	SDS [10 %]
	50 µl	APS [10 %]
	5 µl	TEMED
2X SAMPLE BUFFER	4 % (w/v)	SDS
	20 % (v/v)	Glycerol
	120 mM	Tris pH 6.8
	10 % (v/v)	β-mercaptoethanol
	0.02% (w/v)	Bromophenol blue

SDS-RUNNING BUFFER	25 mM	Tris-base
	192 mM	Glycine
	0.1 % (w/v)	SDS
STACKING GEL	1.4 ml	dH_2O
	0.33 ml	Acryamide [30 %]
	0.25 ml	Tris-HCl [1 M] (pH 6.8)
	20 µl	SDS [10 %]
	20 µl	APS [10 %]
	2 µl	TEMED

Table 16: Solutions, kits and instruments for protein biochemical experiments

Solutions and other material	Company
Acrylamide [30 %]	Biorad
APS	Serva
BSA	Sigma
β-mercaptoethanol	Serva
Centrifugation tubes; Quick seal	Beckmann
Colloidal gold-nanoparticles (15 nm)	BBInternational
Coomassie Brilliant Blue R-250	Sigma
ECL	Thermo Scientific
IPTG	Applichem
Fibronectin (human)	Milipore
Flexible dialyse tube (12 000 MWCO)	Serva
Gluthation-Sepharose 4 Fast Flow	GE Healthcare

Hyperfilm	GE Healthcare
Latex beads, 3 µm (LB-30)	Sigma
Milk powder	Sucofin
Nitrocellulose membrane	Biorad
PageRuler™ Prestained Protein Ladder	Fermentas
PMSF	Applichem
ProteinA-coated gold-nanoparticles (15 nm)	BioCell
SDS	Roth
TEMED	Biorad
Tween-20	Applichem
Vivaspin 2	Sartorius

Instruments	
FACscalibur	Becton Dickinson
FESEM “Merlin”	Zeiss
French-Press; SLM-Aminco, 40.000 PSI	Fisher
Mini-PROTEAN 3 System	Biorad
Nano drop	Thermo Scientific
RC5C-Centrifuge, rotor: SLA-3000	Sorvall
RC5C-Centrifuge, rotor: SS-34	Sorvall
Rotor TLA 100.2	Beckmann
Ultra centrifuge TL-100	Beckmann
TEM EM910	Zeiss

4.6.2 Overexpression and purification of recombinant GST-fusion proteins

Large scale overexpression

Positive *E. coli* transformants (see 4.5.6) were grown over night at 37 °C with agitation (120 rpm) in LB media in the presence of ampicillin. Pre-culture was diluted 1:100 in 500 ml fresh LB media and incubated at 30 °C with shaking until bacteria reached late exponential growth phase ($OD_{600\ nm}$= 0.8). 1 ml of culture was removed for SDS-PAGE analysis, centrifuged at 5000 rpm for 5 min; the pellet was stored at - 20 °C ("*pre-induction*"). 500 ml pre-warmed LB media containing IPTG giving a final concentration of 1 mM to induce expression of GST-fusion proteins was added to the bacteria culture and further incubated over night. 1 ml of culture was removed for SDS-PAGE analysis, centrifuged at 5000 rpm for 5 min and the pellet was stored at - 20 °C ("*post-induction*"). Bacteria were harvested by centrifugation at 8000 rpm for 10 min and resuspended in 20 ml sterile PBS. After another centrifugation step bacteria were homogenously resuspended in 10 ml PBS supplemented with 1 mM PMSF and kept on ice. Cell lyses was carried out by a French-Pressure cell. By centrifugation at 15,000 rpm for 15 min cell debris was removed from the lysate fraction which was finally stored at - 20 °C until further processing. 100 µl of lysate fraction were removed for SDS-PAGE analysis and stored at - 20 °C ("*FP lysate*").

Purification

GST-fusion proteins specifically bind glutathione sepharose (Gluthation-Sepharose 4 Fast Flow). Purification of the recombinant proteins was carried out by GST-affinity-chromatography with glutathione immobilized on a sepharose-matrix. After equilibration with PBS for several times, the matrix was incubated with the bacteria lysate at 4 °C over night. In this way, not GST-tagged proteins and other soluble cellular components were removed. By centrifugation at 500 rpm for 10 min the matrix was separated from the residual lysate from which 100 µl were store at - 20 °C ("*Flow-through*") for SDS-PAGE analysis. The matrix was washed three times with sterile PBS before the GST-tagged recombinant proteins were eluted with 3 ml elution buffer for 1 h by rolling at room temperature and finally dialyzed against PBS over night at 4 °C. 50 µl of GST-fusionprotein solution were store at - 20 °C ("E_d") for SDS-PAGE analysis. Overexpression was analysed by SDS-PAGE (see 4.6.3). Protein concentrations of the different samples were calculated with nano-drop measurement, increased with Vivaspin Concentrators and conformed to defined stock values. Samples were stored at - 20 °C as working stocks and at - 80 °C as long-term backup-stocks.

4.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This method was the first time described by Laemmli (1970) and is used for separation of denatured proteins in an electric field based on their molecular weight. Protein denaturation and dissociation is realized in the presence of SDS and β-mercaptoethanol. Samples were mixed with sample buffer, heated for 10 min at 98 °C, cooled down and applied to the gel matrix. Proteins were collected in the 4 % stacking gel (pH 6.8) and separated in a 10 % resolving gel (pH 8.8). Electrophoresis was run in 1 x SDS-running buffer using the Mini-Protean 3 System; PageRuler Prestained Protein Ladder was used as molecular weight standard.

After SDS-PAGE the proteins were stained with Coomassie R-250 Brilliant Blue. Gel was several times microwave-boiled in the staining-solution and background destaining was done by microwave-based boiling for several times and finally incubation with agitation over night at room temperature in dH_2O.

4.6.4 Fibronectin binding analysis via Dot Blot

Fibronectin-binding capacity of the recombinant SfbI protein constructs was investigated by Dot Blot method. Dot Blot analysis allows detection of functionality of soluble proteins under native conditions.

Different amounts of recombinant protein were spotted on a nitrocellulose membrane. After drying the membrane was incubated in 10 % (w/v) milk in PBST for 1 h at room temperature, before washed once and probed with 20 µg fibronectin in 10 ml PBST for another 1 h, followed by three washing steps and incubation with an anti fibronectin HRP-conjugated antibody in 10 ml PBST for 1 h. After washing the membrane five times, HRP enzyme activity was detected with ECL, and visualized by exposing the blot to a hyperfilm for different time periods. The signal was detected and fixed by incubation the film with appropriate solutions.

All washing steps were carried out with PBST.

4.6.5 Coating of latex beads with recombinant protein

For investigation of adhesion- and invasion-potential of the SfbI derivates 3 µm latex beads were coated with recombinant protein.

1 x 10^8 beads were washed three times with 500 µl PBS by centrifugation at 3000 rpm for 10 min and incubated with 5 mg recombinant protein over night at 4 °C. Beads were washed twice with PBS and subsequently incubated with 10 mg/ml BSA in PBS for 1 h at room temperature. After a final washing step beads were resuspended in 500 µl PBS and enumerated using a Neubauer counting chamber. Coating efficiency was analyzed by fluorescence activated cell sorting (FACS) and electronic microscopy (see 4.6.6).

4.6.6 Detection of surface associated SfbI on GAS and latex beads

GAS A40 and A157 were grown over night in TSB and fixed with 1 % PFA before centrifugation for 10 min at 3500 rpm and resuspended in PBS. Latex beads (4.6.5) and bacteria were incubated with specific anti-SfbI IgG (1:75 in PBS) for 1 h at room temperature and washed twice with PBS by according centrifugation steps.

Electron microscopy

After incubation with the first antibody samples were incubated with ProteinA coated 15 nm colloidal gold-nanoparticles for 30 min at room temperature and washed twice with PBS. 2 µl of the resuspended sample pellets were applied onto nickel grids coated with a butvar-film; samples were allowed to settle for 5 min. Rinsinng of the grids was done with TE-buffer and dH_2O. Samples were analyzed by transmission (EM910) or field emission scanning (Merlin) EM.

FACS

After incubation with the specific anti-SfbI IgG samples were incubated with an Alexa488-conjugated secondary antibody (goat anti rabbit IgG; 1:400 in PBS) for 30 min at room temperature before samples were analyzed using a FACscalibur and the cell quest (BD) software.

4.6.7 Coating of 15 nm colloidal gold with recombinant protein or BSA

The pH of the colloidal gold-nanoparticle sole was adjusted to 6 with 1.1 M K_2CO_3. This step is crucial since it changes the electrostatic charge of the gold-nanoparticles. Negatively charged gold-particles can now be linked to positively charged proteins. Since this interaction is by van der Waals forces the biological activity of the protein is not influenced (schematically depicted in Figure 11); final protein concentration [10 µgml^{-1}].

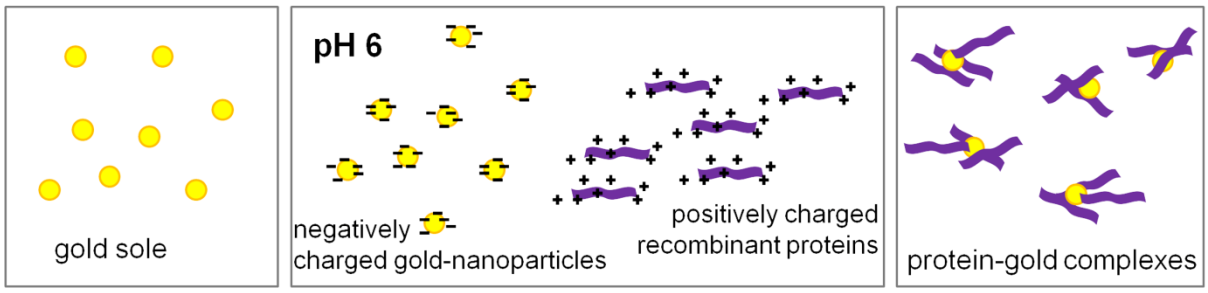

Figure 11: Schematic overview of protein-gold complex formation

Stabilisation of the coated gold-nanoparticles was tested by mixing protein-gold complex solution 1:1 with 10 % NaCl solution. A colour change from red to blue is indicative for an unstable coating of the gold-nanoparticles. Since no colour change was detected the protein-gold solution was concentrated by centrifugation at 20,000 rpm and 4 °C for 20 min. The resulting loose pellets were collected in less than 1 ml fluid. TEM analysis revealed more than 95 % single protein gold-nanoparticles.

4.7 Cell culture

Table 17: Cells and culture media

Cells	Supplier/ reference	Media	Media supplements
PRIMARY HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)	Lonza	EGM-2	SupplementPack Pen/Strep [100 Uml^{-1}]/ [0.1 mgml^{-1}]

LARYNGEAL EPITHELIAL CELL LINE (HEP-2)	ATCC CCL23	DMEM	10 % FCS 2 mM l-Glutamine Pen/Strep [100 Uml^{-1}]/ [0.1 $mgml^{-1}$]

Table 18: Media, solutions and instruments for cell culture experiments

Media and solutions	**Company**
EGM-2	PromoCell
SupplementPack	PromoCell
Pen/Strep	PAN Biotech GmbH
DMEM (low glucose)	Gibco
Trypsin/EDTA	PromoCell/ PAN Biotech GmbH
HEPES-BSS	PromoCell
l-Glutamine	PAN Biotech GmbH
FCS (10 %; heat inactivated)	PAA
Glass cover slides (12 mm diameter)	Menzel
Instruments	
Light microscope, Axiovert 25	Zeiss
CO_2 incubator	Thermo Scientific
Centrifuge	Heraeus

Cells were cultured in specified media (for details see Table 17) in 10 cm cell culture dishes at 37 °C and in a 5 % CO_2 atmosphere. Cells were passaged when reaching 90 % confluency; for this purpose the cell layer was washed with HEPES (HUVEC) or PBS (Hep-2) to remove cell debris and media left overs, before the cells were detached from the plastic surface using 3 ml trypsine/EDTA. This process was stopped by adding 7 ml of the according cell culture media before HUVECs were subsequently collected by centrifugation for 10 min

at 900 rpm, resuspended in 4 ml fresh media and ~ 4 x 10^5 cells were seeded in fresh media on new culture dishes. Hep-2 cells were seeded respectively but without a centrifugation step and without antibiotics.

For co-incubation and infection assays cells were seeded onto glass cover slides within 24-well plates. 500 µl media were placed per well, before 4 x 10^4 cells/well (HUVEC) or 3 x 10^4 cells/well (Hep-2) were added. Co-incubation-/ Infection-assays used for IF were performed with 75 % confluent cell layer, for inhibition-assays an established confluent monolayer was developed.

4.8 Preparation and procedure of infection and co-incubation assays

Table 19: Media and buffer for co-incubation and infection assays

CACODYLATE BUFFER	100 mM	Cacodylate
	90 mM	Sucrose
	10 mM	$MgCl_2$
	10 mM	$CaCl_2$
EM-FIXANS	5 %	PFA
	2 %	Glutaraldehyde
	0.1 M	Cacodylat-buffer
TSB		See Table 7

Table 20: Solutions and instruments for co-incubation and infection assays

Solutions	**Company/ reference**
EBM	PromoCell
Gentamycin	Sigma
Saponin ([1 %] in PBS)	Merck
DMEM (low glucose)	Gibco
PFA ([4 %] in PBS)	USB
PFA ([25 %] in dH_2O)	Sigma
Calphostin C	Enzo
Edelfosine	Calbiochem
FCS (10 %; heat inactivated)	PAA
Methyl-β-cyclodextrin	Sigma
BAPTA-AM	Calbiochem
Penicillin G Sodium (1610 U/mg)	Sigma
Glutaraldehyde [37,5 %]	Merck
Trypsine/EDTA	PAN Biotech GmbH
Instruments	
Photometer (Novaspec II)	Pharmacia
Light microscope Axiovert 40 C	Zeiss
CO_2 incubator	Thermo Scientific
Centrifuge 5804 R	Eppendorf
37 °C incubator	Haereus

4.8.1 Co-incubation of HUVEC with protein-gold complexes

BSA-gold loading of HUVEC lysosomes

Lysosomes were pre-loaded by feeding HUVEC with BSA-gold-nanoparticles (15 nm). A confluent HUVEC-layer in a 6 cm-culture dish was incubated with 150 µl BSA-gold solution (see 4.6.7) in 4 ml standard cell culture media under standard conditions (for details see 4.7). The following day, cells were co-incubated with 1 x 10^7 beads coated with recombinant protein (see 4.6.5) and fixed after 4 h in EM-fixans and stored at 4 °C until further preparation.

Co-incubation of HUVEC with recombinant protein-gold complexes

HUVECs were seeded on glass cover slides in a 24 well cell culture plate and incubated under standard conditions (see 4.7). When reaching ~ 80 % confluency cells were co-incubated with recombinant protein-gold complexes for 45 min by adding 30 µl/ well protein-gold solution. Incubation was stopped by fixation in EM-fixans and stored at 4 °C until further preparation.

4.8.2 Co-incubation of HUVEC and recombinant protein coated latex beads

Standard approach

HUVECs were seeded on glass cover slides in a 24 well cell culture plate and cultured under standard conditions (see 4.7). When reaching ~ 80 % confluency cells were co-incubated with 1 x 10^6 latex beads coated with recombinant protein (see 5.6.5) per well for 45 min at 37 °C and 5 % CO_2 after culture plates were centrifuged for 5 min at 500 rpm. By this centrifugation step the coated latex beads and the cellular surface were brought in close proximity leading to synchronized invasion events.

Cellular signaling inhibition studies

For analysis of signaling pathways involved in SfbI-induced uptake of beads or streptococci HUVECs were washed once with EBM lacking serum compounds and subsequent pretreated with 5 mM MBCD (suspended in culture media), 8 µM BAPTA-AM, 1 µM calphostin C or edelfosine (all compounds suspended in DMSO) for 30 min, before proceeding respectively to the standard approach (see above). MBCD influences lipid rafts

and was shown to inhibit caveolae-dependent internalization; BAPTA-AM chelates intracellular Ca^{2+} and thus was shown to influence phagocytic uptake processes. Another required component in the signal transduction pathway during phagocytosis is the protein kinase C (which was shown to be inhibited by calphostin C. Edelfosine, was shown to target the membrane associated pospholipase C which is a further essential member of phagocytic signaling networks. The drugs remained present during the entire incubation period. Finally the samples were fixed in 1 % PFA followed by microscopic analysis. The percentage of intracellular beads in the drug-samples was compared to the number of intracellular beads of the respective media control with or without DMSO.

4.8.3 Infection of HUVEC with GAS

GAS A40 and A157 were grown over night in TSB at 37 °C (see 4.4). Streptococci were harvested by centrifugation at 3500 rpm for 10 min. Bacteria were resuspended in PBS and the bacteria solution was adjusted to a $transmission_{600nm}$ of 10 % before 1:200 diluted in EBM supplemented with 5 % FCS.

HUVEC were seeded on glass cover slides and cultured up to 80 % confluency. Before cells were infected with GAS for defined time periods they were twice washed with antibiotic free EBM; finally 500 µl of the adjusted bacteria suspension was placed per well. Infection was controlled by light microscopical observations and was fixed with 1 % PFA at defined time points and stored at 4 °C until further preparation. Incubation was realized under standard growth conditions.

4.8.4 Infection of Hep-2 with GAS

GAS A40 and A157 were grown over night in TSB at 37 °C (see 4.4). Streptococci were harvested by centrifugation at 3500 rpm for 10 min. Bacteria were resuspended in PBS and the bacteria solution was adjusted to a $transmission_{600nm}$ of 10 % before they were diluted 1:200 in DMEM supplemented with 10 % FCS.

Hep-2 cells were seeded on glass cover slides and cultured up to 80 % confluency in antibiotic free cell culture media. Media was replaced by 500 µl of the adjusted bacteria suspension per well. After different time periods of incubation with standard parameters and controlled by light microscopical observations samples were fixed with EM-fixans and stored at 4 °C until further preparation.

4.8.5 Survival studies of SGO inside Hep-2 cells

Infection of Hep-2 with SGO

SGO heterologous expressing GfbA, GfbApro, SfbI or SfbIGaro were inoculated over night in TSB at 37 °C in the presence of the specified antibiotics (see 4.4).

SGO were harvested by centrifugation at 3500 rpm for 10 min. Bacteria were resuspended in PBS and the bacteria solution was adjusted to a $transmission_{600nm}$ of 10 % before 1:200 diluted in DMEM supplemented with 10 % FCS.

Hep-2 cells were seeded on glass cover slides and cultured up to 80 % confluency in antibiotic free cell culture media. Media was replaced by 500 µl of the adjusted bacteria suspension per well and samples were incubated under standard conditions for 1.5 h. Cover slips were washed once with media and one sample was taken for the following plating step whereas the others were subsequent placed into media supplemented with 10 % FCS and with the antibiotics penicillin [5 $\mu g ml^{-1}$] and gentamycin [100 $\mu g ml^{-1}$] killing all extracellular bacteria and were further incubated for defined time periods.

Plating of cell-associated SGO

Cover slips were washed twice with PBS before infected cell were detached from the glass surface by 100 µl/well trypsine/EDTA. By adding 400 µl/well saponin solution cells were gently permeabilized for 20 min at 37 °C. Finally serial dilutions of culture-suspension were plated on THY-agar and surviving cfu were enumerated after growth at 37 °C for ~ 24 h. The percentage of surviving SGO after different time periods was compared to the number of viable cell-associated bacteria before antibiotic treatment.

4.9 Microscopical techniques

Table 21: Media and buffer for microscopical experiments

TE BUFFER (pH 7.0)	20 mM	TRIS
	1 mM	EDTA
CACODYLATE BUFFER		See Table 19
PBS		See Table 15

Table 22: Solutions, materials and instruments for microscopical experiments

Solutions and materials	Company/ reference
Acetone	J. T. Baker
Agar ([2%]; w/v; aq)	Difco
Butvar ([1 %]; w/v in chloroform) B98	Agar Scientific
Cu-grids	Plano
Immersol 518N	Zeiss
Gelatin capsules, operculatea No. 2	Pharmapol
osmium tetroxide [1%] (aqueous)	Roth
Pb-citrat (Ultrastain 2)	Leica
Spurr resin	Sigma/ Fluka
Triton X-100	Sigma
UAc ([2 %]/ [4 %]; w/v)	Serva

Instruments	
Centrifuge (Mikro rapid)	Hettich
Critical Point Dryer (CPD 030)	Baltec
Diamond knife	RMC
TEM EM 910	Zeiss
FESEM DSM 982 Gemini	Zeiss
FESEM Merlin	Zeiss
Filter sets (No.02, 09, 15)	Zeiss
Sputter coater SCD 500	BalTec
Ultratrimm, Reichert	Leica
Ultramicrotome, Reichert Ultracut	Leica
ZeissAxiophot	Zeiss
ZeissAxiocam HRc digital camera	Zeiss

4.9.1 Electron microscopy (EM)

4.9.1.1 Field emission scanning electron microscopy (FESEM)

Electron microscopy of this type direct-images the surface of the sample by detecting secondary and backscattered electrons resulting from the interaction of the electron beam and the sample. Beam electrons are emitted by a field emission gun and interaction signals are collected by a specific detector (Everhart-Thornley detector). These signals are translated by a computer into an image reproduction depicted on a screen.

In the present study on the one hand the interactions of HUVECs with beads or gold-nanoparticles coated with recombinant protein were investigated on the other hand the internalization mechanisms of GAS into Hep-2 cell were analyzed.

The fixed samples (see 4.8.1 and 4.8.4) were washed twice with TE buffer before dehydrating in a graded series of acetone (10 %, 30 %, 50 %, 70 %, 90 %) on ice for 10 min

each step. Samples were incubated twice in 100 % acetone for 15 min at room temperature before subjected to critical-point drying with liquid CO_2.

Dried samples were covered with a gold-palladium film by sputter coating (GAS infection on Hep-2; 4.8.4) or for visualization of protein-coated gold-nanoparticles (4.8.1) samples were evaporated with carbon and finally examined in a field emission scanning electron microscope (DSM 982 Gemini) using the Everhart Thornley SE detector and the SE-inlens detector with an acceleration voltage of 5 kV or in the field emission scanning electron microscope (Merlin) using the SE-inlens detector and the Energy Selective Backscattered electron (EsB) detector with an acceleration voltage of 5 kV for visualization of gold-nanoparticles. The EsB-detector was used for the detection of material contrast and allows the detection of small gold-nanoparticles on the cell surface or already after internalization under the cellular membrane. In addition, the EsB-detector offers the possibility to discriminate between false-positive signals and positive gold signals. Brightness, intensity and contrast were adjusted with Corel Photo-Paint X6.

4.9.1.2 Transmission electron microscopy (TEM)

TEM creates translucent images of ultrathin samples. An electron gun on top of the microscope emits electrons which are further focused to a beam subsequently interacting with the sample. Dependent on the sample depth the electrons are more or less scattered; electrons passing the sample reach the bottom of the microscope where they are visualized as a signal on a fluorescence screen or by a camera. According to the density of the material the sample is depicted as a “shadow-image” in various grey shadings. During the preparation process samples need to be contrasted by insertion of different heavy metals because biological structures typically have very low own contrast. Furthermore, special attention must be turned to ultrathin sectioning since thickness of the analyzed sample is a leading factor for TEM analysis and for the quality of the resulting image.

After several washing steps with cacodylate buffer, the samples (see 4.8.1) were secondary fixed and in parallel pre-contrasted with 1% aqueous osmium tetroxide for 1 h at room temperature, and subsequently washed with cacodylate buffer. Cells were scrapped off the cell culture dish and pelleted at 5000 rpm for 3 min before immobilized in 2 % Agar.

Samples were dehydrated in a graded series of acetone (10 %, 30 %, 50 %, 70 %, 90 %) on ice for 30 min each step including a contrasting step in 70 % acetone/ 2 % UAc over night at

4 °C and were finally incubated twice in 100 % acetone for 30 min at room temperature before embedded in gelatin capsules in Spurr resin according to described procedures (Spurr, 1969); resin was polymerized for 10 h at 70 °C.

By trimming of the embedded specimen ultrathin sectioning was prepared. 80 - 90 nm thick sections cut with a diamond knife and collected from the water surface with butvar-stabilized Cu-grid. Specimens were post-contrasted with 4 % UAc (aq) for 3 min and in a CO_2-reduced atmosphere with Pb-citrate for 2 min. Finally sections were washed with dH_2O to remove remaining staining material and air dried before being analyzed in a TEM EM 910.

Images were recorded digitally with a Slow-Scan CCDCamera (ProScan) with ITEM-Software (Olympus Soft Imaging Solutions). Brightness, intensity and contrast were adjusted with Corel Photo-Paint X6.

4.9.2 Immune fluorescence (IF) microscopy

IF microscopy is a standard technique for the detection of proteins which are either directly stained with a fluorochrome-tagged substance (i.e. phalloidin or FITC-tagged IgG) or first marked with a specific IgG and then stained with a secondary fluorochrome-tagged IgG recognizing the first antibody. A specific wavelength form light of a high-pressure mercury vapor lamp is selected by several filter sets (Table 23, excitation) and by the fluorochromes on the specimen. These fluorochromes emit light with a longer wavelength than the previously excited light. The emitted wavelength is again selected by filter sets removing any scattered light (Table 23; emission).

Table 23: Filter sets for IF microscopy

FILTER SET	EXCITATION	EMISSION	FLUOROCHROME
No. 02	G365	LP 420	DAPI
No. 09	BP 450 - 490	LP 515	Alexa Fluor® 488
No. 15	BP 546/12	LP 590	Alexa Fluor® 568

After fixation samples (see 4.8.2 and 4.8.3) were washed twice with PBS before specifically labeled due to the different experimental set ups. For details concerning the used antibodies see Table 6. All incubation steps were performed at room temperature; in between the incubation steps the glass cover slides were washed twice with PBS. Finally the samples were mounted in ProLong® Gold Antifade Reagent with Dapi, sailed with clear nail polish and stored at 4 °C in the dark until analysis.

Label of SfbI on extracellular beads

Extracellular protein-coated beads were labeled using an anti-SfbI antibody incubated for 60 min, followed by an Alexa® Fluor488-conjugated secondary antibody for 30 min.

Label of the actin cytoskeleton

After permeabilization with 0.1 % Triton X-100 for 3 min the actin cytoskeleton was stained with Alexa® 568-conjugated phalloidin for 30 min.

Label of Arp2/3 complex

After permeabilization with 0.1 % Triton X-100 for 3 min Arp2/3 complex was stained with an anti-ArpC1b antibody for 60 min and an Alexa® Fluor488-conjugated secondary antibody for 30 min.

Label of cellular and bacterial DNA

Dapi a fluorescent dye that strongly binds DNA molecules is included within the mounting reagent. Therefore, no additional staining step was necessary.

For IF analysis ZeissAxiophot with an attached ZeissAxiocam HRc digital camera and Zeiss Axiovision software 8.2 were used. Brightness, intensity and contrast were adjusted with Corel Photo-Paint X6.

4.10 Statistic analysis

Data were analyzed with Excel 2000 (Microsoft). Each experiment was performed in triplicate in least three independent experiments. Mean and standard deviation were calculated from all independent experiments and compared to their respective controls. Differences were analyzed using two-tailed, paired Student's t-test; $p < 0.05$ was considered significant.

5. RESULTS

5.1 GfbA, SfbI and their derivates induce different entry processes into host cells and recombinant proteins trigger distinct integrin-clustering

It has already been shown that recombinant SfbI protein tagged to gold-nanoparticles interacts with host cell $\alpha_5\beta_1$-integrins via Fn resulting in integrin-clustering which is detectable by aggregated gold-nanoparticles on the host cell surface (Rohde *et al*., 2003). Since the invasion mechanisms triggered by GfbA, SfbI and their derivates GfbApro lacking the AroD region, and SfbIGaro a chimeric SfbI construct in which the AroD region was replaced by the homologue region of GfbA, show different morphologies it could be speculated that the effect on cellular integrins might also be affected. Therefore, HUVECs were incubated with gold-nanoparticles, coated with the different recombinant proteins.

Integrin-clustering indicated by aggregations of gold-nanoparticles on the cellular surface and the uptake of gold-aggregates by large invaginations in the cell membrane were observed by high resolution field emission scanning microscopy (FESEM) when HUVECs were incubated with those recombinant proteins which were shown to induce bacterial entry by co-opting caveolae i.e. SfbI and GfbApro (Figure 12). GfbA and SfbIGaro shown to cause cytoskeleton rearrangements when heterologous expressed on SGO were unable to trigger detectable integrin-clustering or caveolae-aggregation, instead membrane alterations on the cellular surface were induced and only single or pair-wise gold-nanoparticles were found (Figure 12).

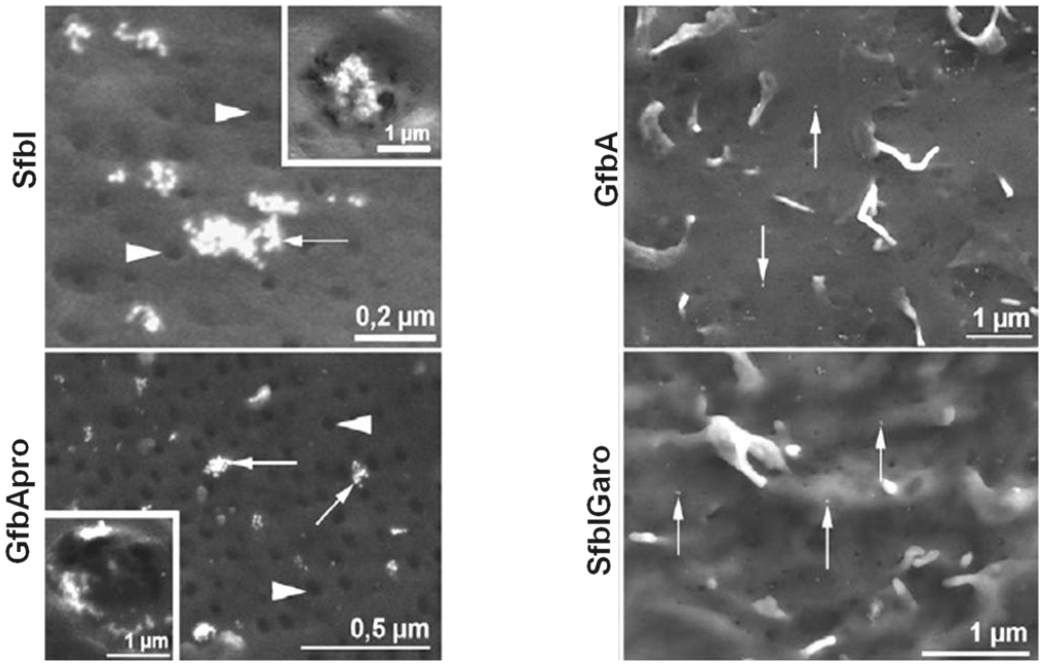

Figure 12: Incubation of HUVEC with recombinant protein-coated gold-nanoparticles. SfbI and GfbApro trigger aggregation of caveolae (SfbI, GfbApro, arrowheads) and aggregation of gold-nanoparticles indicative for integrin-clustering (arrows) and uptake of gold aggregates by HUVEC cells (inserts), GfbA and SfbIGaro-coated gold-nanoparticles induce membrane alterations, but exhibit no caveolae aggregation and no gold-nanoparticle clustering, and therefore no integrin-clustering, only small gold aggregates are detectable (arrows).

These findings strongly suggest that the AroD region of GfbA contains *e.g*. structural information that modulates the entry process induced by GfbA as well as by SfbI when containing the GfbA-AroD, by preventing integrin-clustering and triggering cytoskeleton rearrangements.

5.2 Invasion mechanisms-dependent survival rates of SGO heterologous expressing the different FnBPs

Since Rohde and his collaborators observed that the intracellular trafficking route of streptococci strongly correlates with the invasion mechanism the question which needed to be answered was if trafficking along the alternative pathway benefits streptococcal survival. Therefore, Hep2 cells were infected with SGO heterologous expressing the GfbA, SfbI and their derivates (see above) for defined time periods, antibiotics were added to kill

extracellular bacteria before finally the cells were lysed, survived SGO were plated and cfu were enumerated the following day. Figure 13 shows the survival rate of the different SGO after four, eight and 24 h. While more than 50 % of the intracellular SGO SfbI and SGO GfbApro survive within the first 24 h after caveolae-dependent invasion and following intracellular the alternative pathway, the entry via cytoskeleton rearrangements and trafficking along the phago-lysosomal route results in a significant loss of survival and persistence of the SGO expressing GfbA or SfbIGaro. This effect is most properly due to the fusion of bacterial compartments with cellular lysosomes and subsequent degradation of the bacteria.

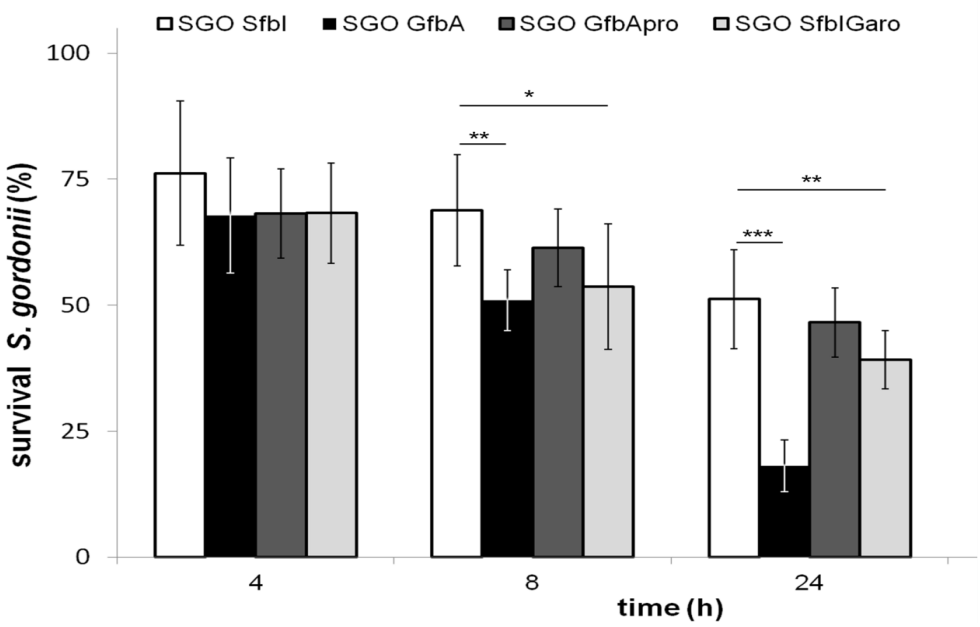

Figure 13: Intracellular survival rates of SGO expressing heterologous SfbI, GfbA or their derivates on the surface. Within the first 24 h after caveolae-dependent invasion more than 50 % of the intracellular SGO SfbI and SGO GfbApro survive whereas the entry via cytoskeleton rearrangements results in a significant loss of survival of the SGO expressing GfbA or SfbIGaro. Survival rates were calculated by enumerating cfu after defined time periods compared to start point when antibiotics were added The graphs show mean values of at least three independent experiments, error bars indicate ±SD of the means (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

In conclusion the obtained results on the function of the AroD region of the Fn-binding proteins of GAS (SfbI) and GGS (GfbA) lead to the hypothesis that AroD modulates the early steps of the entry process by regulating integrin-clustering which might lead to distinct invasion mechanisms resulting in a corresponding intracellular trafficking route and subsequently affecting the bacterial survival.

5.3 Dependent on the No. of ProD repeats GAS strains invade host cells in distinct ways

Clinical GAS isolates which were already identified for expressing different SfbI proteins by Towers *et al*. (2003) were analyzed in terms of their invasion into host cells and the underlying uptake mechanism. A broad range of morphological differences not only including the expected caveolae-dependent internalization of streptococci (Figure 14, left pictures GAS strains A 40, A 310, A 184, white arrowheads), but also actin cytoskeleton rearrangements involved in streptococcal uptake were observed for a number of isolates despite expressing SfbI on the surface (Figure 14, right pictures, GAS strains A 157, A 171, A 316 (kindly provided by M. Rohde), white arrows).

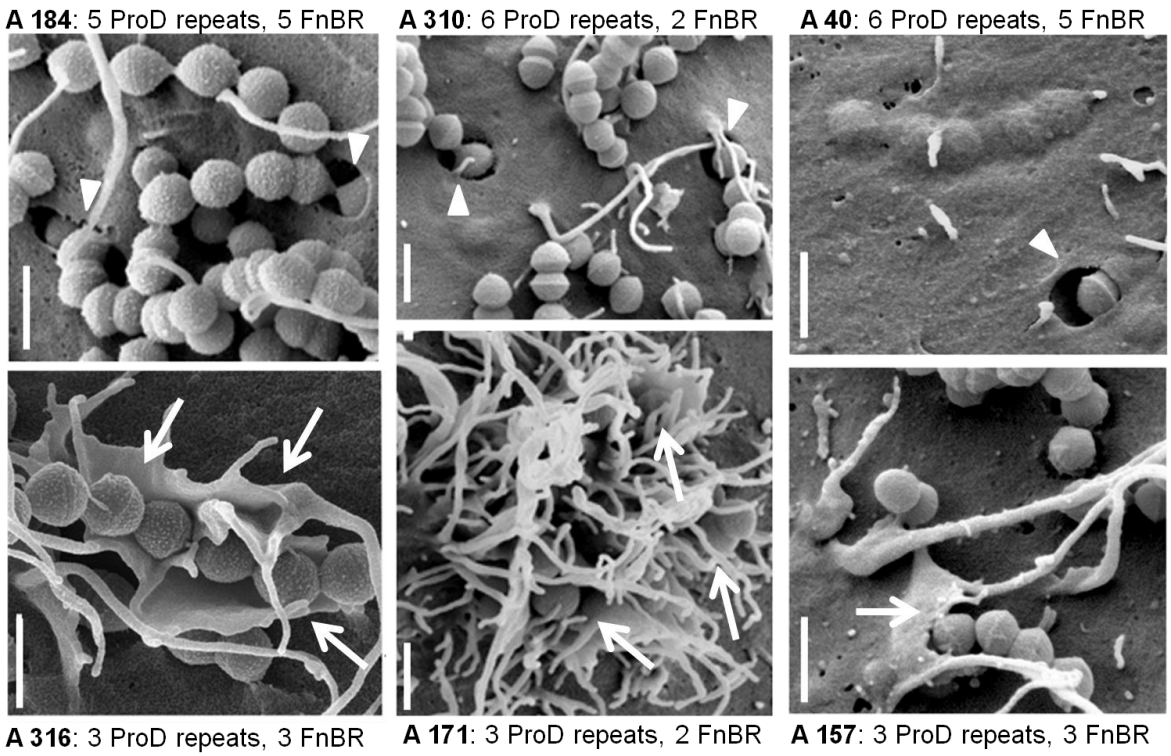

Figure 14: Different SfbI-induced uptake mechanisms based on the protein structure. Hep2 cells were co-incubated with clinical GAS isolates expressing different SfbI constructs for defined time periods. FESEM revealed that dependent on the number of ProD repeats and FnBRs GAS induce morphological distinct uptake processes. The GAS strains A40, A310 and A184 expressing SfbI with high number of ProD repeats/ FnBRs, trigger caveolae-dependent uptake mechanisms (white arrowheads), whereas GAS strain A157, A171 and A316 (picture kindly provided by M. Rohde) expressing a low number of ProD repeats and FnBRs, invade host cells by inducing actin cytoskeleton rearrangements (white arrows). Bars represent 1 µm.

The distinct SfbI-dependent invasion mechanisms could be attributed to the number of the ProD repeats and of the FnBRs. Either high number of ProD repeats or of FnBRs or of both trigger streptococcal uptake by caveolae-aggregation and the formation of large invaginations (GAS strains A40, A310, and A184, Figure 14), whereas low number of ProD repeats and of FnBRs induce actin cytoskeleton rearrangements (GAS strains A157, A171, and A316, Figure 14). Furthermore, it could be investigated that ProD effects the actin cytoskeleton by inducing stress fiber degradation when heterologous expressed on SGO (Branitzki-Heinemann *et al*., in preparation).

Cytoskeleton rearrangements were so far usually attributed to M protein mediated invasion processes of GAS (reviewed by Nitsche-Schmitz *et al*., 2007) and to GfbA, the SfbI-homologue of Group G streptococci (GGS), induced uptake of GGS (see above, Rohde *et al*., 2011). These results revealed for the first time that dependent on the modular structure, i.e. number of ProD repeats and FnBR of SfbI, different streptococcal invasion mechanisms can be triggered.

5.4 Nucleotide sequence of the *sfbI* gene from GAS strain A40 and of SfbI-constructs generated by inverse PCR technique

Using the SfbI protein of the *S. pyogenes* strain DSM2071, Talay and her colleagues investigated the modular domain structure in detail (1994). In 2003 Towers *et al*. analyzed the *sfbI* genes of numerous clinical GAS isolates and showed how variable the domain structure is distributed among *S. pyogenes* strains.

Since the specific effects of the ProD should be analyzed it was decided to generate *sfbI*-constructs with different number of ProD repeats but with identical N- and C-terminal backbone for not altering the function of the AroD and Fn-binding regions in SfbI. To do so, the *sfbI* gene from the GAS strain A40, used in the studies of Rohde *et al*. was first of all sequenced. In Figure 15.A the complete nucleotide sequence of the *sfbI* gene from GAS A40 is shown. The deduced SfbI protein structure consists of five FnBRs at the C-terminus, an N-terminal AroD and six repeats of the ProD in the central part (underlined nucleotides indicate the beginning of the repeats). The ProD repeats three, four, five and six have nearly identical primary sequences, whereas repeat one and two show divergent structures with 50 % and 85 % identity, respectively. The alignment to the *sfbI* gene sequence with *S. pyogenes* DSM2071 SfbI shows high identity of the repeats, except that the *sfbI* (GAS A40) gene

encodes for six ProD repeats. The N-terminal aromatic acid rich regions reveal 73 % homology.

Based on the *sfbI* gene sequence from the GAS A40 strain, *sfbI* constructs were generated by inverse PCR technique using the pGEX-cloning system. Primer pairs were chosen to receive constructs lacking different number of ProD repeats but consist of identical N- and C-terminal parts (without signal sequence and membrane anchor).

The challenge consisted in deleting ProD repeats with identical nucleotide sequences and therefore usage of the same primer pairs. Precise adjustment of annealing temperature, extension time and template were critical for the experimental outcome and high numbers of transformed *E. coli* colonies were screened for the correct inserts since the inverse PCR generated most often a heterogenic mixture of ***sfbI*** ***∆ProD repeats*** constructs. One out of one hundred *E. coli* clones could carry the designated construct, while the other carried just *e.g*. the original template sequence. In summary, this successful cloning strategy resulted in the six ***sfbI*** ***∆ProD repeats*** constructs. In Figure 15.B the peptide sequences of the ProD of the SfbI from GAS A40 (first lane), *S. pyogenes* strain DSM2071 (second lane) and the ***sfbI*** ***∆ProD repeats*** constructs (lane three to eight) are depicted. As already mentioned above, six ProD repeats are found within the SfbI from GAS A40 and four repeats in the case of *S. pyogenes* strain DSM2071 (the underlined prolines indicate the beginning of each repeat). The SfbI ∆ProD repeats constructs feature five to zero ProD repeats (SfbI ∆1- SfbI ∆5 abbreviated in different number of repeats; SfbI ∆ProD lacking the whole ProD region).

All constructs generated in the present study consist of different number ProD repeats, but share identical N- and C-terminal nucleotide sequences with the parental SfbI of GAS A40; nucleotide sequences were checked before continuative experiments.

Figure 15.A: Nucleotide sequence of the *sfbI* gene from GAS strain A40 and alignment with an already sequenced *sfbI* from another well-studied GAS strain. Sequence alignment of the complete *sfbI* gene from *S. pyogenes* strain DSM2071 (upper lane, Talay *et al*., 1994) and the clinical isolate *S. pyogenes* A40 (lower lane). Identical residues are marked with an asterisk, the underlined nucleotides indicate the beginning of the ProD repeats.

Figure 15.B: Alignment of the peptide sequence of the ProD from different SfbI-proteins. Amino acid consensus sequences of the *sfbI-ProD* regions obtained by sequence analysis of the *sfbI* gene from *S. pyogenes* A40 (upper lane), *S. pyogenes* strain DSM2071 (second lane) and the different ***sfbI*** ***∆ProD*** constructs (lane three to eight, generated in the present study). Conserved prolines within the ProD are shown in bold and the underlined prolines indicate the beginning of the ProD repeats, identical residues are marked with an asterisk.

A

B

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GAS A40     SAEYVPDDPPKPGDTSEHNPKTPELDGTPIPEDPKRPDESSEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 194
DSM2071     SAEFV----------PENPPKPGETPEHGPKTPELDGTPIPEGPQRPNESLEPTLPPVMLDGQEVPEVPSESLEPALPPLMPELDGQEVPEVPSESLEPALPPLMPELDGQEVPEKPSVDLPIEDPRYEFNNKDQSPLAGESGETEYITEVYGN 144
Δ1          SAEYVYV----------PEDPKRPDESSEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 171
Δ2          SAEYVYV----------  ----------PEDPKRPDESSEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 147
Δ3          SAEYVYV----------PEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 120
Δ4          SAEYVYV----------PEVPSESLEPALPPLMPELDGEEVPEVPSESLEPALPPLMPELDGEEVPEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 96
Δ5          SAEYVYV----------PEVPSESLEPALPPLMPELDGEEVPEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 72
ΔProD       SAEYVYGYV----------PEKPSVDLPIEVPRYEFNNKDQSPLAGESGETEYITEVYGN 50
            ***:*
```

5.5 SfbI constructs show comparable Fn-binding capacity

SfbI is the major Fn-binding protein for adhesion and the main invasin of GAS. The molecular basis for its binding to the ECM molecule Fn is well characterized (Schwarz-Linek *et al*., 2003; 2004; 2006). Schwarz-Linek *et al.* conclusively demonstrated that each repeat of the C-terminal FnBR binds to a Fn molecule thereby opening the RGD-region of Fn which subsequently binds to host cell $\alpha_5\beta_1$-integrins (Talay *et al*., 2000).

Since the different SfbI-constructs in the present study share the same C-terminal Fn-binding region the Fn-binding capacity of the different recombinant SfbI-proteins was assessed by dot blot analysis. Proteins, recombinant overexpressed as GST-SfbI-fusion-proteins in *E. coli*, were purified and immobilized on a nitrocellulose membrane.

As shown in Figure 16 all constructs bound human Fn to comparable amounts. The Fn-binding was concentration dependent and did not result from the GST-tag, as GST alone did not bind Fn. In general, the strongest signal was detectable for 1 µg of spotted protein (Fig.5.5).

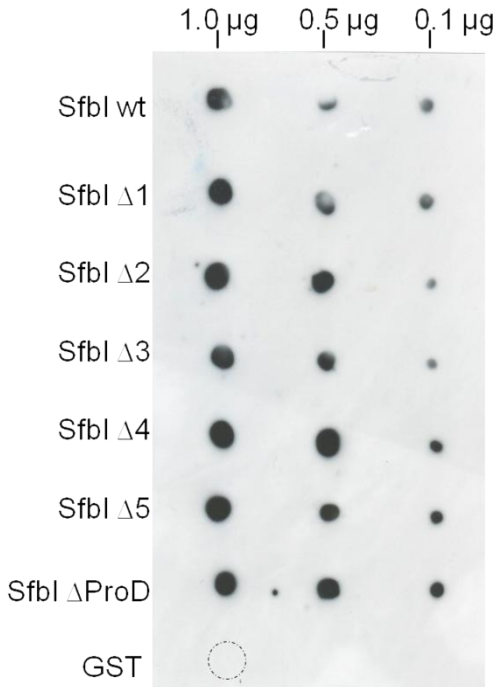

Figure 16: Different SfbI-constructs have comparable fibronectin binding capacity. SfbI constructs bound fibronectin in a concentration-dependent manner to similar amounts. Proteins were spotted on a nitrocellulose membrane; after blocking, the blot was probed with human fibronectin and a goat anti-fibronectin-HRP-conjugated antibody. HRP enzyme activity was detected with ECL, and visualized by exposing the blot to a film.

Thus, a distinct effect of the different SfbI constructs within following co-incubation experiments does not depend on the binding efficiency of Fn.

5.6 Polystyrene latex beads coated with different SfbI constructs invade host cells

As the Fn-binding capacity of the SfbI-constructs was not affected, we next investigated whether the different recombinant SfbI-constructs still harbor invasive characteristics. Polystyrene latex beads were coated with purified recombinant GST-SfbI-fusion proteins and co-incubated with HUVECs, after fixation samples were labeled for actin and SfbI and analyzed. Coating efficiency was assessed by FACS-analysis and by immune gold label followed by scanning electron microscopical analysis (for representative visualization the SfbI wt and ΔProD were depicted in Figure 17).

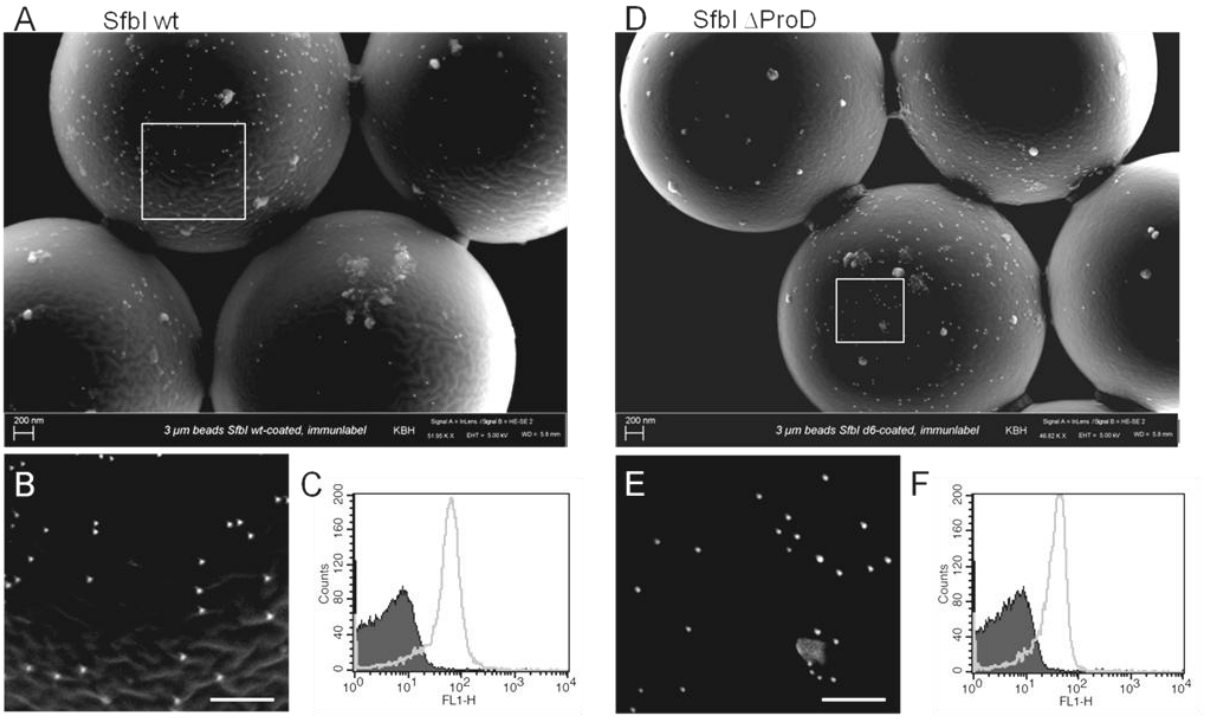

Figure 17: Coating efficiency of Polystyrene latex beads, coated with SfbI wt or SfbI ΔProD. 1x10^{8} latex beads were coated with 5 µg recombinant protein. Coating efficiency was detected by gold-immune label and subsequent FESEM analysis or by fluorescence label and FACS analysis. SfbI wt and ΔProD show comparable coating pattern: FESEM revealed a slight heterogenic population of beads coated with the recombinant protein (A, B); B and E are enlarged sections of A and B, respectively; each white dot represents a single gold-particle detecting SfbI protein; bars represent 200 nm. Depicted in C and F are representative histograms of flow cytometric measurements of fluorescence labeled SfbI-protein on the surface of latex beads (light grey lane) compared to unlabeled beads control (black line).

Over 80 % of all cell-associated beads were internalized, independent of the SfbI-protein on the bead-surface (Figure 18). SfbI wt (Figure 18.A) and SfbI ΔProD (Figure 18.B) are depicted for representative visualization of the protein-capacity to efficiently induce bead-internalization. Since GST alone did not induce uptake of the GST-coated beads (Figure 18.C) it can be assumed that the uptake is an induced process dependent on the recombinant SfbI protein coated to the surface of the latex beads.

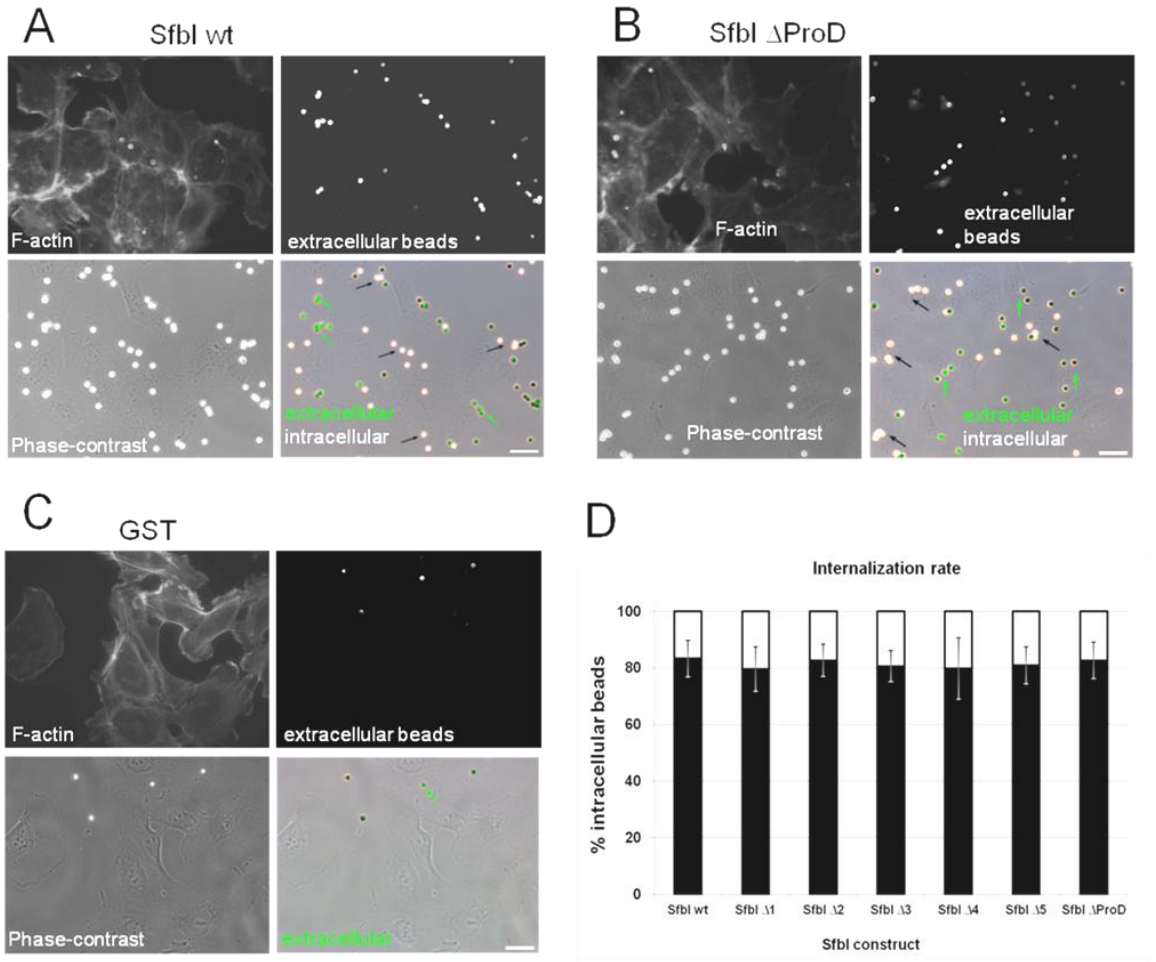

Figure 18: Polystyrene latex beads, coated with different SfbI-constructs, highly invade HUVECs with similar internalization rates. Time-dependent, SfbI wt (A) and SfbI ΔProD (B) coated beads invaded HUVECs to similar amounts, whereas GST (C) coated beads showed no internalization. HUVECs were co-incubated with distinct number of polystyrene latex beads for 2 h; after fixation extracellular beads (green; *green arrows*) were labeled with an anti-SfbI (anti-GST, respectively) first antibody and a Alexa-488 conjugated secondary antibody to differentiate extra- from intracellular beads (*black arrows*). Bars represent 20 µm.

D: Bead internalization rates were quantified by enumerating the ratio of intracellular beads from all cell-associated beads (mean values ± SD of three independent experiments).

These results demonstrate the crucial role of SfbI as a main streptococcal invasion-factor into endothelial cells, but do not clarify the role of the ProD repeats during the invasion process, since it made no difference for bead internalization efficiency if the recombinant SfbI protein contain numerous or no ProD repeats.

5.7 ProD inhibits cytoskeleton rearrangements but cause caveolae-aggregation and actin-accumulation closely around invading beads

The results strongly indicated that ProD influences the interaction of SfbI with the host cell surface and with the actin cytoskeleton subsequently modulating the SfbI-dependent invasion mechanism. For that reason the uptake process of the SfbI-coated beads were analyzed in more detail by elucidating the role of the actin cytoskeleton at the port of entry. Remodeling of the actin cytoskeleton, especially branching of the actin fibers, is realized by the heptameric actin-related protein 2/3 (Arp2/3) complex which plays a central role in cytoskeletal dynamics by controlling actin-filament nucleation (Campellone *et al*., 2010). Therefore, a possible involvement of the Arp2/3 complex in the invasion process was investigated. HUVECs were co-incubated with protein-coated beads and after fixation analyzed according to the different aspects by FESEM or FM.

The recruitment and the fusion of caveolae were detectable at sides of attachment of recombinant SfbI wt-coated beads and HUVECs (Figure 19.A, white arrows). Beads closely accumulated actin (Figure 19.C), but not the Arp2/3 complex (Figure 19.E). Staining of the Arp2/3 complex around the coated beads was similar to the general Arp2/3 staining pattern within the host cell. Arp2/3 complex could be found aggregating around the area around the entry port, however, no direct association with the beads could be observed. In addition, the F-actin staining showed no formation of a membrane-like structure around the bead implicating that no triggering of actin-branching was induced (Figure 19.A/C).

In contrast to recombinant SfbI wt protein, the recombinant ΔProD construct induced clearly detectable actin-cytoskeleton rearrangements around the invading beads, visualized by FESEM (Figure 19.B) and F-actin labeling with fluorescent phalloidin (Figure 19.D). Aggregation of caveolae was not found on the cellular surface around adherent beads (Figure 19.F). Most prominent was the identical labeling pattern of actin and the Arp2/3 complex around invading beads coated with the ΔProD construct resulting in the formation of a membrane-like structure around the beads.

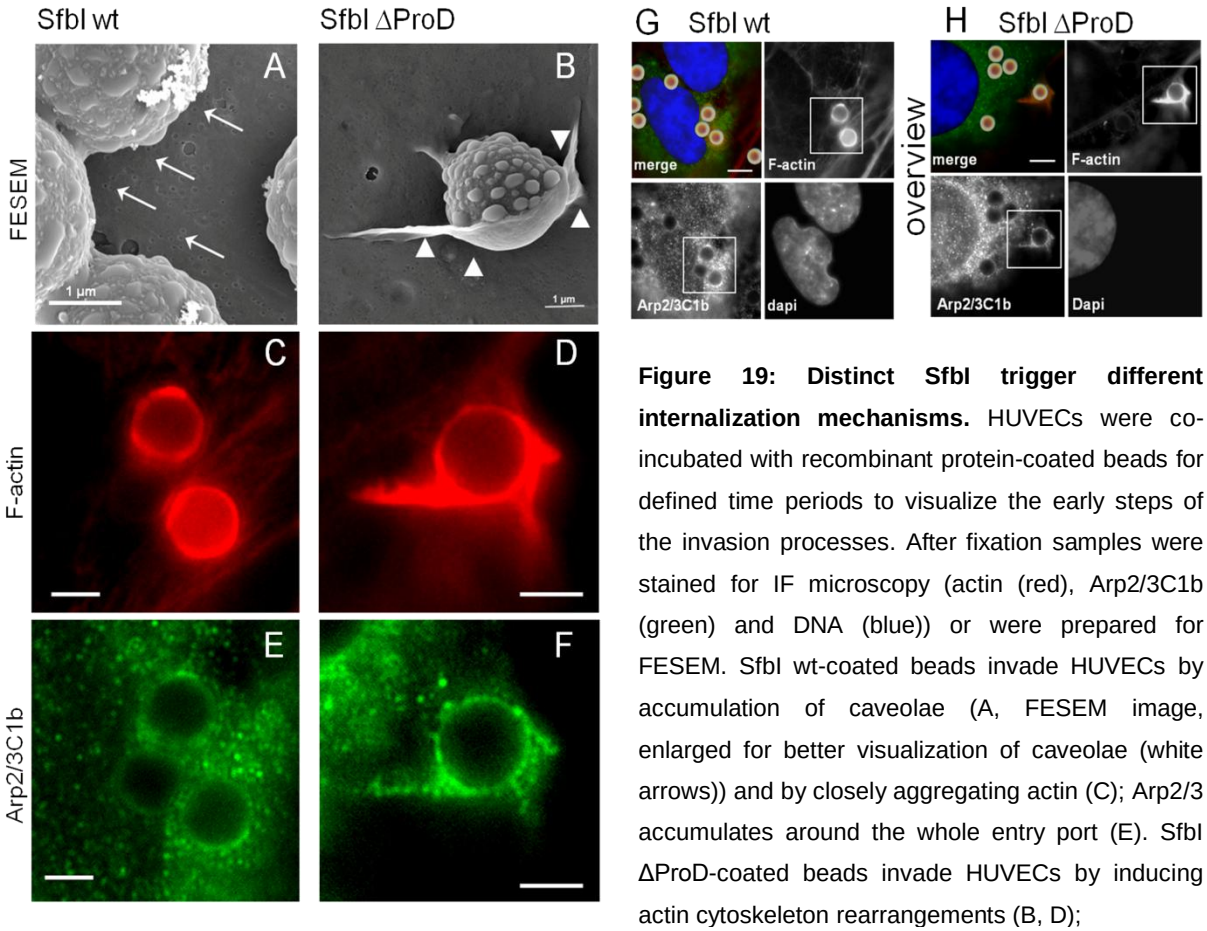

Figure 19: Distinct SfbI trigger different internalization mechanisms. HUVECs were co-incubated with recombinant protein-coated beads for defined time periods to visualize the early steps of the invasion processes. After fixation samples were stained for IF microscopy (actin (red), Arp2/3C1b (green) and DNA (blue)) or were prepared for FESEM. SfbI wt-coated beads invade HUVECs by accumulation of caveolae (A, FESEM image, enlarged for better visualization of caveolae (white arrows)) and by closely aggregating actin (C); Arp2/3 accumulates around the whole entry port (E). SfbI ΔProD-coated beads invade HUVECs by inducing actin cytoskeleton rearrangements (B, D); Arp2/3 aggregation occurs codependent (F). C and D are enlarged sections from the according F-actin channel, E and F are enlarged sections from the according Arp2/3C1b channel. G and H are overview pictures of the IF staining. If not otherwise specified, bars represent 2µm (enlarged figures) or 5 µm (overview figures).

These observations gave evidence that the ProD repeats might interact with the Arp2/3 complex during the invasion process and therefore play a prominent part in the invasion pattern of the SfbI-induced internalization process. A high number of ProD repeats prevent Arp2/3 complex-controlled branched actin cytoskeleton rearrangements and, therefore, favors the caveolae-mediated invasion which is not dependent on branched actin rearrangements.

5.8 Different actin- and Arp2/3 complex-pattern during GAS invasion

Since the immune fluorescent microscopy data revealed distinct actin- and Arp2/3- pattern for the SfbI-induced uptake of polystyrene latex beads as a function of the number of the ProD repeats it was now investigated if clinical SfbI expressing GAS isolates varying in the number of ProD repeats and FnBRs influence actin cytoskeleton rearrangements by interacting with Arp2/3 function in the host cell..

HUVECs were infected with GAS A40 which expresses SfbI consisting of six ProD repeats and five FnBRs, or with GAS A157 which expresses SfbI consisting of three ProD repeats and three FnBR. Comparable SfbI expression was visualized by immune-gold-labeling of the SfbI protein on the surface of the *S. pyogenes* isolates analyzed by scanning and transmission electron microscopy (Figure 20).

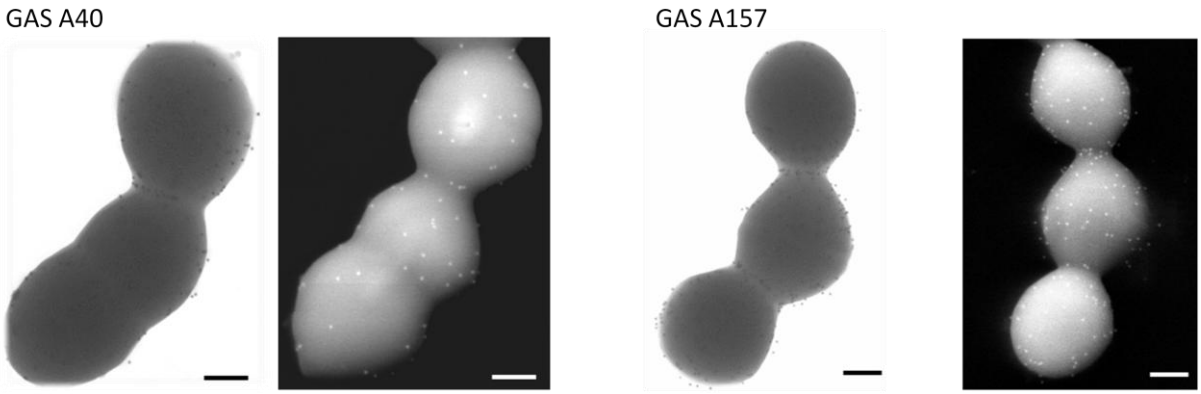

Figure 20: SfbI expression analysis by gold-immune label and electron microscopical analysis. SfbI protein of GAS strain A40 and A157 were labeled with gold-nanoparticles after anti-SfbI antibody detection. The left images show TEM data, the right images show FESEM results. EM revealed SfbI protein distributed over the streptococci and a slightly higher SfbI-expression of GAS A157 compared to the A40 strain. Depicted are representative images, each black (TEM images) or white (FESEM images) dot represents a single gold-particle detecting SfbI protein; bars represent 200 nm.

As depicted in Figure 21.A (merged panel, enlarged sections from according channels) F-actin accumulation and Arp2/3 complex recruitment was closely linked to the invading streptococci belonging to the A40 isolate, indicating interaction of the ProD repeats with the Arp2/3 complex but preventing the formation of membrane-like structures around the invading GAS A40 chain. In contrast, GAS A157 predominantly induced large cytoskeleton

rearrangements and only few aggregated Arp2/3 complexes were found being associated within (Figure 21.B; merged panel, enlarged sections from according channels).

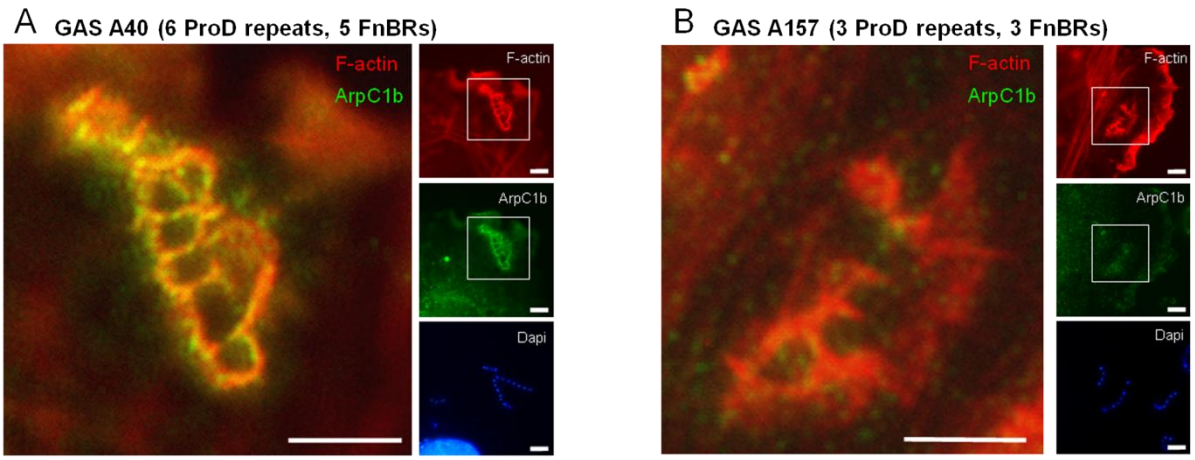

Figure 21: GAS invade HUVECs with distinct F-actin and Arp2/3 pattern. HUVECs were co-incubated with GAS for defined time periods to visualize the early steps of the invasion process. After fixation samples were stained for IF microscopy (actin (red), Arp2/3C1b (green) and DNA (blue)). Fluorescence microscopy revealed a distinct accumulation of F-actin and Arp2/3 in the vicinity of invading GAS A40 (A). GAS A157 invade HUVECs by inducing actin cytoskeleton rearrangements; sparse Arp2/3 aggregation occurs codependent (B). Merged pictures are depicted as enlarged sections from the according F-actin and ArpC1b channel, bars represent 5 µm.

Taken together the data demonstrate that SfbI recombinant expressed and coated on polystyrene latex beads, as well as protein expressed on the surface of clinical *S. pyogenes* isolates modulates the uptake process into endothelial host cells by causing distinct actin- and Arp2/3 complex-pattern due to interaction of the ProD repeats with the Arp2/3 complex. Thus, resulting in morphologically distinct invasion patterns, i.e. high number of ProD repeats trigger caveolae-driven invasion by SfbI-expressing GAS whereas low numbers of ProD repeats favor invasion via cytoskeleton rearrangements since the Arp2/3 complex is fully functional for branching actin.

5.9 Lack of ProD prevents integrin-clustering on the host cell surface

It has already been shown that SfbI induces the aggregation of caveolae at the point of streptococcal entry, leading to the formation of large invaginations on the host cell surface (see above; Molinari *et al.*, 2000; Rohde *et al*., 2003; 2011). Binding of SfbI to host cell $\alpha_5\beta_1$-integrins via Fn results in integrin-clustering and a distinct uptake mechanism. To gain insights into the details of the invasion mechanism and a possible difference based on the number of ProD repeats, HUVECs were incubated with gold-nanoparticles, coated with different recombinant SfbI, wt and the ΔProD construct. After coating more than 99 % sole SfbI-gold particles were found in the protein-gold solution (Figure 22).

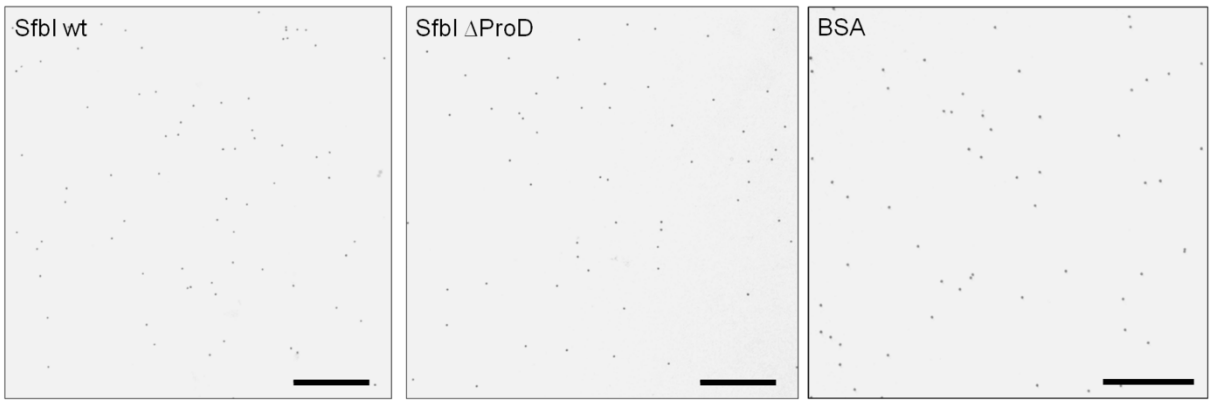

Figure 22: Protein gold-nanoparticles do not aggregate. TEM data revealed more than 98 % single protein tagged gold-nanoparticles. Depicted are representative images, each black dot represents a single gold-particle; bars represent 500 nm.

Analysis by high resolution field emission scanning microscopy (FESEM) with respect to integrin-clustering, which is detectable by aggregated gold-nanoparticles on the host cell surface revealed that HUVECs incubated with recombinant wt SfbI show gold-nanoparticles aggregations on the surface and the uptake of those aggregates by large invaginations in the cell membrane (Figure 23.A, Inlens EsB detector, white arrows). SfbI-gold aggregates were taken up within large surface cavities (Figure 23.A, InLens SE-Detector channel, dashed circles). In contrast, gold-nanoparticles bound to recombinant SfbI ΔProD, were still detectable as mostly single protein-gold complexes, though in close proximity but not aggregating. This suggests that SfbI ΔProD still interacted with the host cell surface but did not induce any integrin-clustering. Expectedly, the detectable intracellular protein-gold complexes were not associated with cavity-like structures (Figure 23.B).

In general, most gold-nanoparticles were already found internalized under the cell surface, giving no clear signal with the InLens SE-Detector (Figure 23.A and B, right channel). The InLens EsB-Detector (Figure 23.A and B; left channel), however, revealed a clear pattern of gold-nanoparticles.

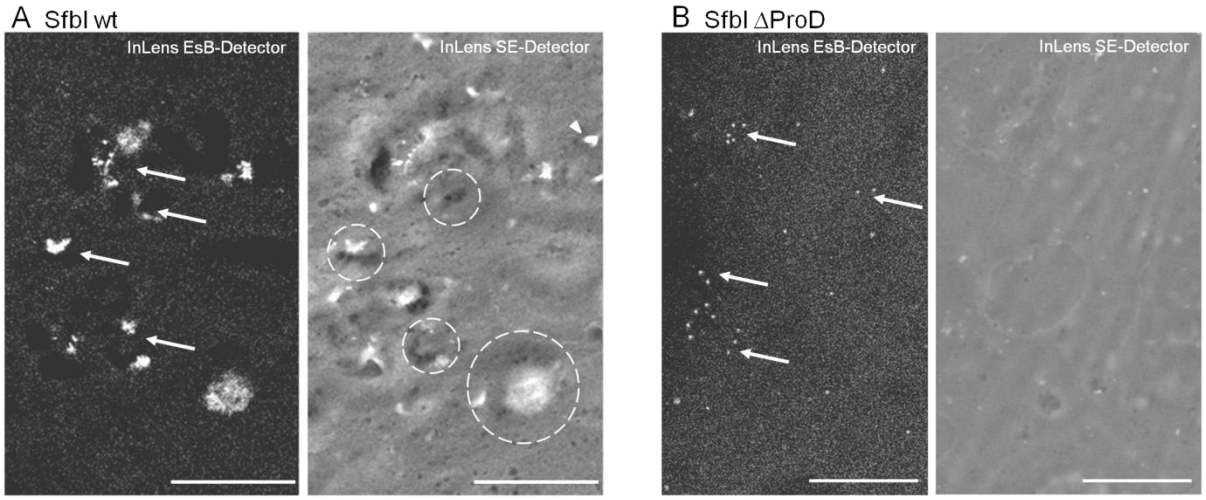

Figure 23: Dependent on the coated SfbI, gold-nanoparticles show distinct clustering patterns on the surface of HUVECs. In addition to the InLens SE-Detector, the InLens EsB-Detector was used for increased material contrast. The EsB-Detector allows the detection of small gold-nanoparticles, already internalized and residing intracellularly, and offers the possibility to discover false-positive signals (*e.g.* A, InLense SE-Detector channel, white arrowhead). A: Full-length SfbI (wt) caused integrin-clustering, indicated by remarkable aggregation of the gold-nanoparticles on the surface of HUVECs (InLens EsB-Detector channel, white arrows). SfbI-gold aggregates were taken up within huge surface cavities (InLens SE-Detector channel, white dashed circles). B: SfbI ΔProD-coated gold-nanoparticles exhibit no clustering, and were still detectable in single protein-gold complexes. Though gold-nanoparticles were found internalized under the cell surface, any small cavity-like structures were not associated with gold-nanoparticles. Bars represent 2 µm.

These findings give strong evidence that the number of ProD repeats plays also a role by modulating the invasion process during SfbI-mediated uptake by triggering integrin-clustering. A low number of ProD repeats prevents detectable integrin-clustering and subsequent formation of invaginations, leading to a different uptake mechanism via actin cytoskeleton rearrangements.

5.10 Different signaling cascades play a role in the distinct invasion mechanisms

Different invasion processes might be regulated by different intracellular signaling events; therefore it was assessed how the caveolae-based endocytic uptake, blocking of intracellular Ca^{2+} or the inhibition of the phospholipase C or of the protein kinase C affects the number of internalized protein-coated latex beads.

Methyl-β-cyclodextrin (MBCD) is the most efficient member of the group of cyclic glucose oligomers, capable of selectively capturing cholesterol from membranes and thus destroying caveolae structures (Klein *et al.*, 1995; Yancey *et al*., 1996; Hailstones *et al.,* 1998; Parpal *et al*., 2001). MBCD was shown to prevent caveolae-based endocytosis of salmonella (Lim *et al*., 2010) and of nanoparticles (Hao *et al*., 2012) and of caveolae-dependent SfbI-induced uptake of GAS (Rohde *et al*., 2003). Pre-treatment of HUVECs with 5 mM MBCD inhibited SfbI wt-based internalization of latex beads via caveolae to more than 60 % (Figure 24.A; black bar), whereas the internalization of beads coated with SfbI ΔProD, which triggers cytoskeleton rearrangements, was significantly less affected (40 % inhibition) (Figure 24.A; white bar).

Microscopical analysis of SfbI ΔProD-induced uptake of latex beads and GAS expressing SfbI with low number of ProD repeats and FnBRs revealed entry processes showing typical features of classical phagocytosis such as formation of membrane protrusions and massive F-actin accumulation. The Intracellular calcium ($[Ca^{2+}]_i$) chelator BAPTA-AM was shown to strongly inhibit phagocytosis by decreasing the amount of free $[Ca^{2+}]_i$ (Kobayashi *et al.*, 1995; Edberg *et al.*, 1995; Peracino *et al*., 1998; Seastone *et al*., 1999; Cohen *et al*., 2006). $[Ca^{2+}]_i$ is involved in the signal transduction during phagocytotic uptake processes and is thus next to others essential for efficient phagocytic activity (Nunes and Demaurex, 2010). Another required component in the signal transduction pathway during phagocytosis is the PKC which can be inhibited by calphostin C (Karimi, and Lennartz, 1998; Larsen *et al.,* 2000; 2002; Mancuso and Peters-Golden, 2000; Tardif *et al*., 2002). Pre-treatment of HUVECs with 8 µM BAPTA-AM or 1 µM calphostin C reduced the internalization of SfbI ΔProD-coated latex beads more than 50 % (BAPTA: 52 %; Figure 24.B; calphostin C: 73 %; Figure 24.C; white bars), whereas these substances effect the invasion rate of beads coated with SfbI wt to a significant lower extent (BAPTA: 32 %; Figure 24.B; calphostin C: 42 %; Figure 24.C; black bars).

Another substance, edelfosine, was shown to target the membrane associated PLC which is a further essential member of phagocytic signaling networks by inducing indirectly the release of $[Ca^{2+}]_i$ (Nunes and Demaurex, 2010). Pre-treatment of HUVECs with 20 µM edelfosine cause ~ 75 % reduced internalization rates independent of the coated SfbI (Figure

24.D). Edelfosine not only inhibits the PLC and therefore inactivates phagocytic mechanisms, it also removes cholesterol from membranes (van Blitterswijk and Verheij, 2008; Hac-Wydro *et al*., 2011) and therefore might influence directly caveolae-dependent uptake processes. Thus the influence of edelfosine on the internalization rates of SfbI wt- and SfbI ΔProD-coated beads is multifunctional and, although effecting different targets, might be the reason for the similar inhibitory effect.

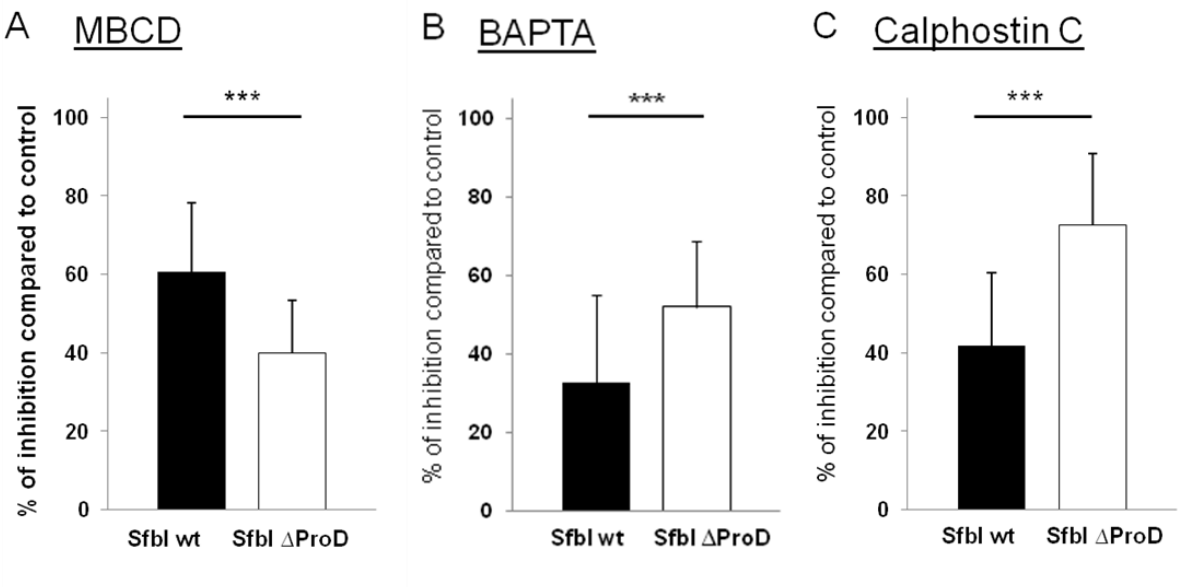

Figure 24: Different signaling cascades are involved in the internalization processes of beads coated with different SfbI. HUVEC were pre-treated for 30 min with the indicated substances specifically inhibiting processes which are involved in the uptake mechanisms of GAS. Afterwards, cells were co-incubated with protein-coated beads for 45 min. The internalization efficiency of beads coated with SfbI wt was largely reduced by MBCD (61 %; A), whereas lower influence on the internalization rate was detectable after treatment with calphostin C (42 %; C) or BAPTA (32 %; B). MBCD showed the lowest inhibitory effect on the internalization of SfbI ΔProD-coated beads (40 %; A), followed by BAPTA (52 %; B); calphostin C (73 %; C) highly blocks bead internalization. Edelfosine inhibited bead internalization more than 70 % independent of the SfbI protein (D). Bead internalization rates were quantified by enumerating the ratio of intracellular beads from all cell-associated beads. The graphs show mean values of three independent experiments, error bars indicate SD of the means (***, $p < 0.001$; n.s. not significant).

These results confirm the previous morphological based findings that SfbI proteins with different number of ProD repeats trigger distinct uptake processes, since the invasion-underlying signaling events could be affected with different inhibitory substances which were triggered by the different modular composition of the recombinant SfbI proteins and the natively expressed SfbI proteins on the surface of different clinical isolates used. Different inhibitors showed different effects on the invasion capabilities tested in our studies.

5.11 ProD inverts the intracellular trafficking route

It was finally of interest if the intracellular fate of the protein coated beads is linked to the entry process since it could be shown that bacteria are localized within caveosomes preventing lysosomal fusion after entry into host cells via the aggregation and subsequent fusion of caveolae which was found to be induced by SfbI and GfbApro. In contrast GfbA and SfbIGaro trigger bacterial uptake via large cytoskeleton rearrangements followed by trafficking intracellular along the classical endocytic pathway with final lysosomal fusion leading to the degradation of the streptococci (see above). HUVECs were pre-incubated with BSA-gold-nanoparticles which were internalized via a receptor-mediated endocytic process. In this way lysosomal compartments were preloaded with gold-nanoparticles before co-incubated with latex beads coated with SfbI wt or SfbI ΔProD, thus, a possible lysosomal fusion could be investigated on ultrathin sections by transmission electron microscopy. TEM analysis strongly revealed co-localization of gold-particles and beads coated with SfbI ΔProD after an entry-process via cytoskeleton rearrangements (Figure 25; black arrows) whereas SfbI wt coated beads were mainly found in compartments in proximity but not fused with lysosomes (Figure 25; white arrows), although some co-localization events were detectable (Figure 25; black arrows).

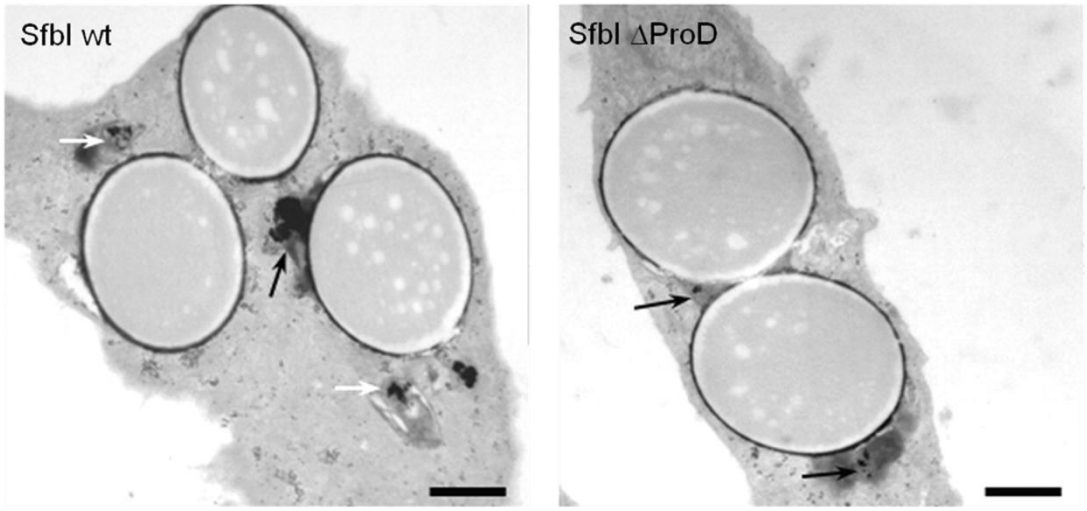

Figure 25: Internalized beads, coated with different SfbI-proteins show distinct colocalisation characteristics with lysosomal compartments. For pre-loading of lysosomes, HUVECs were fed with BSA gold-nanoparticles and subsequently co-incubated with latex beads for 4 h; after fixation samples were prepared for TEM and ultrathin sections were analyzed. Electron microscopic analysis showed fusion of SfbI ΔProD-coated beads with BSA-gold pre-loaded lysosomal compartments (black arrows). The majority of beads, coated with SfbI wt-protein, however, stayed separated from gold containing compartments (white arrows). Bars represent 1 µm.

In summary, these findings give strong evidence that a high number of ProD repeats in the SfbI protein of GAS prevents a phagocytic-like internalization of streptococci and of latex beads coated with recombinant SfbI by triggering integrin-clustering and subsequent intracellular signaling events which induce the caveolae-mediated invasion pathway and therefore favors the fate of bacteria and beads.

5.12 The FnBR are important but not essential for successful adhesion and internalization

The ProD is not necessary for SfbI-dependent internalization since four FnBR and the AroD seemed to be sufficient to induce internalization of beads coated with the SfbI ΔProD lacking the six ProD repeats. However, ProD might not only modify the invasion process but also might support invasion characteristics on a limited scale. Very preliminary data showed that

truncation of the FnBR of the GAS A40 SfbI protein (SfbI ΔFBR) recombinant expressed and coated on latex beads led to reduced but still significant adherence (approximately 70 % compared to the SfbI wt) and invasive (approximately 30 % of adherent beads; cf. SfbI wt: 80 %) capacities (Figure 26.A), although no Fn-binding was detectable anymore (Figure 26.C). Furthermore, the AroD region is supposed to support the adherence and invasion characteristics to some extent since latex beads coated with recombinant SfbI lacking the FnBR and the AroD, thus, just consisting of the ProD, showed still significant adherence (approximately 50 % compared to the SfbI wt) and invasive capacities (approximately 15 % of adherent beads; cf. SfbI wt: 80 %), though, in a lower range than SfbI ΔFBR coated beads (Figure 26.B).

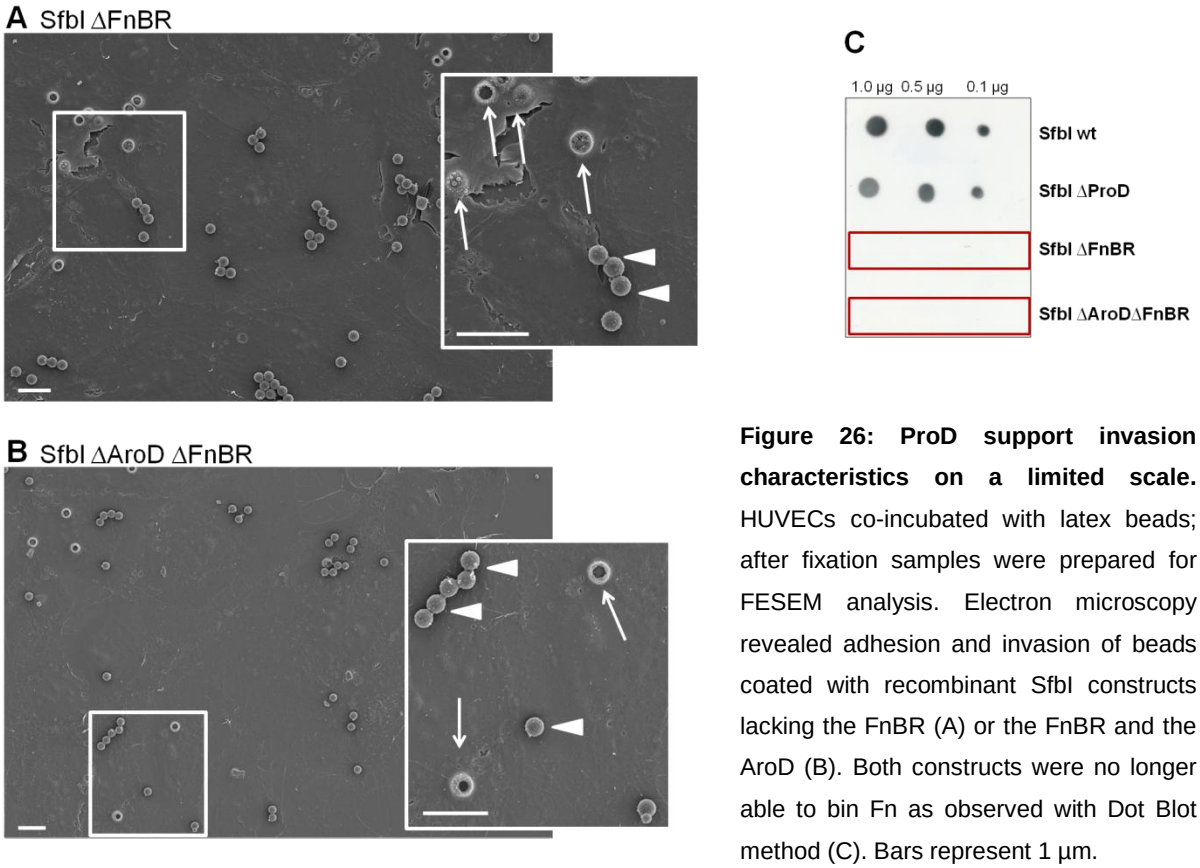

Figure 26: ProD support invasion characteristics on a limited scale. HUVECs co-incubated with latex beads; after fixation samples were prepared for FESEM analysis. Electron microscopy revealed adhesion and invasion of beads coated with recombinant SfbI constructs lacking the FnBR (A) or the FnBR and the AroD (B). Both constructs were no longer able to bin Fn as observed with Dot Blot method (C). Bars represent 1 µm.

These results confirm the hypothesis that the AroD region somehow, i.e. by conformational changes, modifies the interaction of the ProD repeats and the SfbI in general with the host cell surface, i.e. integrins, and that the ProD functions not only as a response of the FnBRs during the SfbI-induced entry process. However, continuative experiments have to be performed before a solid argumentation is possible.

6. DISCUSSION

GAS subvert the various immune defense mechanisms of host cells by several different strategies including invasion of host cells. Internalization might aid evasion of host defense, such as antimicrobial peptides, antibody recognition and phagocytic cleavage, or antibiotic treatment by allowing bacteria to hide inside host cells. To successfully combat GAS infections understanding of streptococcal uptake processes is highly important since different invasion mechanisms might change the fate of intracellular bacteria and therefore might influence the treatment strategies and the infection outcome.

Bacterial uptake into host cells can be realized through the interaction with molecules of the extracellular matrix bridging to receptors on the cellular surface. Fibronectin is a prominent binding partner of bacterial adhesion and invasion factors i.e. Fn-binding proteins SfbI of GAS.

In the current model for SfbI-mediated streptococcal uptake into host cells SfbI associates with $\alpha_5\beta_1$-integrins through a Fn bridge. Since a single SfbI protein can bind several Fn molecules (Schwarz-Linek *et al.*, 2003), this might provoke high amounts of Fn on the streptococcal surface which subsequently trigger integrin clustering on the host cell surface and signaling events that lead to recruitment of caveolae and the formation of large invaginations mediating streptococcal internalization (Rohde *et al.*, 2003; 2011). By co-opting the caveolae-mediated invasion streptococci benefit their survival by trafficking intracellular inside caveosomes avoiding the classical endosomal-lysosomal route which would lead to lysosomal fusion and subsequent elimination of the pathogen (Bohme *et al.*, 2009; Shin *et al.*, 2001). Binding of Fn not only mediates clustering of integrins but also activates signaling pathways that include phosphoinositide 3-kinase, integrin-linked kinase, paxillin, focal adhesion kinase and Src family kinases (Ozeri *et al.*, 2001; Wang *et al.*, 2006[b]; 2007). However, details of the signaling pathways and involved factors still remain to be identified.

FnBPs of gram-positive bacteria such as SfbI of GAS, its homologue GfbA of group G streptococci, or FnBPA of *Staphylococcus aureus* are similar in their modular architecture and consist of a N-terminal signal sequence, a C-terminal cell wall anchoring motif LPX[T,S,A]G (Marraffini *et al.*, 2006), an aromatic amino acid rich domain, a proline rich

domain and the Fn-binding region all varying in sequence length and composition (Schwarz-Linek *et al.*, 2004). Within SfbI proteins of different clinical GAS isolates the central ProD and the C-terminal region consisting of repeats (ProD repeats and Fn-binding repeats) which are conserved in their sequence but differ highly in their number (Talay *et al.*, 1994; Towers *et al.*, 2003). While the FnBRs and their interaction with Fn molecules to initiate the streptococcal uptake are well characterized no information were available for the function of the AroD and of the ProD regions.

6.1 Structure dependent influence on SfbI-mediated internalization processes

Streptococcus gordonii was used as an *in vivo* expression system by S. Talay to obtain detailed information about the involvement of the ProD on the SfbI-mediated invasion process. It was observed that lack of this region triggered an uptake process based on the rearrangement of the actin cytoskeleton and thus, differed completely from the caveolae-dependent invasion mechanisms of streptococci in the presence of the ProD region (S. Talay; personal communication). Fluorescence microscopy analysis of infections with GAS and SGO expressing SfbI with high numbers of ProD repeats and FnBRs on their surface revealed a degradative effect on the host cell actin cytoskeleton (S. Talay; M. Rohde; personal communication).

A correlation of SfbI-structure, meaning the number of ProD repeats and FnBRs, and the invasion mechanisms was supposed when it was investigated that SfbI proteins with different numbers of repeats induce morphological distinct uptake mechanisms of clinical GAS isolates; not only the already described formation of large invaginations dependent on caveolae clustering were found, but also cytoskeleton rearrangements which were previously described for the M protein-induced uptake of GAS or the GfbA-dependent GGS internalization (Cue *et al*., 1998; Molinari *et al*., 2000; Rohde *et al.*, 2003; 2011). High ProD repeat numbers (more than five) predominantly trigger streptococcal uptake by caveolae-aggregation and the formation of large invaginations, whereas low numbers principally induce actin cytoskeleton rearrangements.

For studying the role of the ProD in SfbI-mediated entry processes more in detail SfbI-constructs were generated lacking different numbers of ProD repeats but sharing identical N- and C-terminal structures not altering the function of the AroD and Fn-binding regions in SfbI. Sequence analysis of the *sfbI* gene of GAS strain A40 which served as template for PCR

approaches revealed six ProD repeats and four FnBRs. The challenge of the inverse PCR experiments consisted in deleting gene fragments with identical nucleotide sequences. Precise adjustment of annealing temperature, extension time and template enabled a successful experimental outcome. Recombinant proteins of all SfbI-constructs were overexpressed and Fn-binding capacity was surveyed before the constructs were tested for adherence- and invasion-capability.

It could be shown that the recombinant protein constructs harbored identical Fn-binding capacity and exhibit similar invasive characteristics on HUVECs independent of the number of present ProD repeats in the SfbI-construct. Thus, the ProD region does not affect the internalization efficiency which rather might be regulated by the FnBRs as it was shown recently for the staphylococcal FnBPA (Edwards *et al*., 2010). However, the morphological distinct entry processes could not be stated in this way.

6.2 The ProD region affects the SfbI-dependent internalization processes

Distinct effects on the host cell actin cytoskeleton during SfbI-mediated internalization processes were supposed to depend on the presence and the structure of the ProD region. Details of the underlying pattern induced by recombinant SfbI wt and SfbI ΔProD tagged to latex beads were studied by co-incubation experiments of host cells with protein coated beads and analyzed by fluorescence microscopy.

It could be observed that beads coated with recombinant SfbI ΔProD protein were internalized by actin cytoskeleton rearrangements. In contrast in the presence of the ProD region the formation of membrane-like structures was inhibited resulting in the close accumulation of F-actin around the invading beads and the aggregation of caveolae in the near vicinity as it could be demonstrated for beads coated with recombinant SfbI wt protein.

Remodeling of cellular actin cytoskeleton structures relies on the heptameric Actin related protein 2/3 complex acting as nucleation factor for F-actin filaments branching off from existing mother filaments (see Figure 27). Arp2/3 complex was shown to play an essential role in cellular actin-based processes such as the formation of membrane protrusions or intracellular vesicle-transfer processes including the motility of endo- and phagosomal compartments. Moreover, Arp2/3 complex was demonstrated to be crucial for the motility of different pathogens like *Listeria monocytogenes*, *Shigella flexneri* or *Rickettsia* species (Gouin *et al.*, 2005; Rotty *et al*., 2013). The activity of the Arp2/3 complex relies on numerous

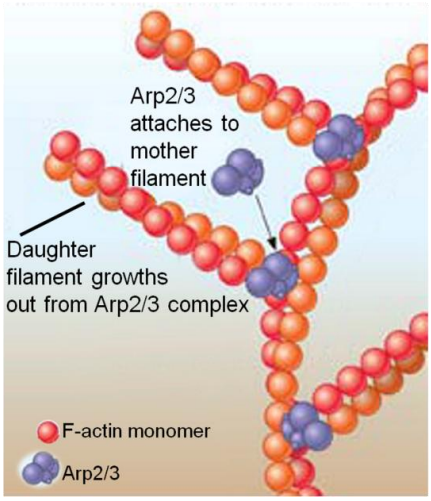

Figure 27: Arp2/3 dependend actin branching (modified from http://photos.thescientist.com/legacyArticleImages/2012/06/06_12_ActinF.jpg)

nucleating promotion factors (NPF) such as WASP (Wiskott-Aldrich syndrome protein), WASP family verprolin-homologous protein (WAVE) or cortactin (Campellone and Welch, 2010; Rotty *et al*., 2013).

A strong correlation of the actin pattern (described above) and the occurrence of the Arp2/3 complex in the case of protein-coated beads but also at entry ports of clinical GAS isolates invading host cells was detectable. These observations gave evidence for an interaction of the ProD with the Arp2/3 complex during invasion processes and lead to the hypothesis that this region might play a prominent role in the invasion pattern of SfbI-induced entry. ProD might prevent Arp2/3 complex-controlled branching of the actin cytoskeleton and favors the caveolae-mediated invasion which is independent of branched actin rearrangements.

Proline rich motifs are commonly found in Arp2/3 activators (Campellone and Welch, 2010); this fact leads to the suggestion that the ProD repeats of the SfbI protein could somehow counterfeit the polyproline motifs of the NPFs. With good reason one may ask the question how SfbI can interact with the cytoplasm-localized Arp2/3 complex. SfbI was found to be secreted by GAS as well as by recombinant expressing SGO (S. Talay, unpublished data). Furthermore, Medina and her colleagues (1999) found that SfbI protein was released within the GAS-containing vacuoles and could be detected in the cytosol of host cells. Thus, SfbI could be able to interact in the cytoplasm with the Arp2/3 complex via its ProD repeats and therefore inhibiting actin branching during internalization processes. Later on, SfbI might modulate intracellular trafficking to prevent lysosomal fusion resulting in a benefit for streptococcal survival. This hypothesis is supported by data of U. Schwarz-Linek who observed direct binding of Arp2/3 subunits with ProD repeats of SfbI by crosslinking experiments. The presence of ProD repeats resulted in the appearance of covalent adducts with apparent molecular weights in the SDS-PAGE analysis of Arp2/3 after crosslinking which were further analysed by MALDI TOF/TOF mass spectrometry of tryptic digests leading to the hypothesis that ProD repeats interact with the Arp2, Arp3 and ArpC1 subunits of Arp2/3 complex (U. Schwarz-Linek, personal communication).

Thus, the ProD region of SfbI not only regulates integrin-clustering and -signaling, moreover, it might function as inhibitor of Arp2/3 complex by direct interaction with distinct subunits; but to verify this argumentation further research has to be done.

6.3 Host cell integrin-clustering due to the ProD region

SfbI-mediated streptococcal uptake into host cells is associated with integrin clustering on the host cell surface; such clustering was also shown to be triggered by recombinant SfbI protein tagged to gold-nanoparticles and was visualized by aggregation of gold-nanoparticles on the host cell surface using field emission scanning electron microscopy (Rohde *et al*., 2003).

In the present study FESEM analysis revealed remarkable accumulation of gold-nanoparticles tagged to recombinant SfbI wt and the internalization of the gold-aggregates within large invaginations, whereas gold-nanoparticles bound to recombinant SfbI ΔProD were still found as mostly single protein-gold complexes. These results give strong evidence that lack of the ProD region prevents detectable integrin-clustering and the subsequent formation of invaginations, leading to a different uptake mechanism. Thus, distinct amounts and/or compositions of host cell integrins might result in different internalization processes.

It is well accepted that SfbI interacts with the host cell surface via the RGD-motif of Fn bridging to $\alpha_5\beta_1$-integrins. However, also other adherence/invasion factors of different Gram positive pathogens, including several M proteins (GAS) or FnBPA and B (*S. aureus*), are well studied for binding Fn and interacting with $\alpha_5\beta_1$-integrins (Nitsche-Schmitz *et al*., 2007; Hoffmann *et al*., 2011). Nevertheless, these proteins do not induce caveolae aggregation and uptake of the pathogen via membrane invaginations.

Indeed, it is not yet finally clarified if there need to be a critical density of Fn leading to host cell integrin-clustering (Schwarz-Linek *et al*., 2006), possibly by high numbers of Fn-binding proteins on the pathogens' surface or by high number of binding repeats within the Fn-binding proteins. It is known that SfbI as well as FnBPA and FnBPB contain high- and low-affinity binding repeats (Schwarz-Linek *et al.*, 2003; 2004; Meenan *et al*., 2007) and it was recently published that in the case of *S. aureus* "[…] *multiple FnBRs increased the speed of internalization* […] *without altering the uptake mechanism* […]". In that study the authors concluded that "[…] *multiple FnBRs within FnBPA facilitate efficient Fn adhesion, trigger rapid bacterial uptake and are required for pathogenesis*." (Edwards *et al*., 2010); though, it needs to be investigated if this is also true for the SfbI-dependent internalization of GAS.

Comparing M1- and SfbI-induced streptococcal uptake Wang *et al*. (2007) attended to the different affinities of M1 or SfbI for Fn since SfbI protein binds to different domains of Fn with greater affinity than M1 protein. This might result in different Fn-binding designs and subsequent distinct integrin-patterns leading to different signaling pathways and finally to a specific streptococcal entry-processes.

Furthermore, other integrin dimers might be involved in the SfbI-mediated uptake of GAS into host cells leading to distinct intracellular signals and effects on the invasion mechanism. For instance, $\alpha_V\beta_3$-integrins were shown to additionally mediate the SfbI-dependent entry of GAS (Ozeri *et al*., 1998) and it is conceivable that other RGD-binding integrins (Humphries *et al.*, 2006) interact with the Fn molecules associated with the pathogens surface dependent on a conformational change in the glycoprotein which is hypothesized to be induced by interaction of Fn with SfbI or FnBPA (Schwarz-Linek *et al*., 2004; Edwards *et al*., 2010). Another possible effect on the integrin-heterogenicity might be the Fn-mediated recruitment of collagen promoted by SfbI and the possible subsequent activation of $\alpha_2\beta_1$-integrins (Emsley *et al*., 2000; Dinkla *et al*., 2003).

These findings and speculations base on the Fn-binding capacity of SfbI, but as the amount of bound Fn is not affected in the SfbI-constructs generated in the present study ProD might cause a structure-dependent conformational change within the SfbI and therefore influence the interaction alternatives of SfbI. Moreover, DGE (Asp-Gly-Glu)-motifs which are demonstrated to be recognized by $\alpha_2\beta_1$-integrins and to be involved in rotavirus entry (Saatz *et al*., 1991; Hewish *et al*., 2000) are present in the repeats of the ProD, except within the first one. It is quite conceivable that the SfbI ProD region directly interacts with $\alpha_2\beta_1$-integrins on the host cell surface and that the activation of those integrins strengthens integrin-clustering and differentiates between caveolae-dependent or cytoskeleton rearrangement-based streptococcal entry mechanisms as a result of distinct signaling cascades.

6.4 The involvement of host cellular signals during distinct uptake mechanisms

Microscopical analysis of SfbI ΔProD-induced uptake of latex beads and GAS expressing SfbI with low number of ProD repeats and FnBRs revealed entry processes showing typical features of classical phagocytosis such as formation of membrane protrusions. Caveolae-dependent or cytoskeleton rearrangement-based streptococcal entry mechanisms might results from distinct host cellular signaling cascades.

The involvement of the small GTPases Rac1 and Cdc42 and the activation of PI3K could already be shown for SfbI-mediated internalization of GAS (Ozeri *et al*., 2001; Purushothaman *et al*., 2003; Wang *et al*., 2007). SfbI-triggered streptococcal uptake furthermore promotes Src-induced phosphorylation of caveolin-1 which up-regulates lipid raft dependent caveolae-endocytosis (Wang *et al*., 2006[b]; 2007; Yamaguchi *et al.*, 2012).

Required components in the signal transduction pathway during professional phagocytotic processes are the phospholipase C which induces the release of intracellular calcium ($[Ca^{2+}]_i$) (Nunes and Demaurex, 2010), $[Ca^{2+}]_i$ then effects another essential member of phagocytic signaling networks the protein kinase C finally regulating the activity of the small GTPases Cdc42 and Rac via activation of GTPases exchange factors (GEFs). Moreover, PKC and the GTPases can directly be targeted by PI3K, and Rac by SRC, respectively (Timpson *et al*., 2001; Welch *el al*., 2003; Beemiller *et al*., 2010). In the end the small GTPases indirectly via the ARP2/3 complex or directly modulate the actin cytoskeleton.

Indeed, there is evidence that different signaling cascades play a prominent role in the distinct invasion mechanisms since it could be observed in the present study that different inhibitory substances affect the internalization rates of beads coated either with recombinant SfbI wt or with SfbI ΔProD to variable extent. The ProD of the SfbI protein might prevent phagocytic-like internalization processes since the number of intracellular beads was significant less reduced by substances inhibiting signals which are known to be associated with phagocytic uptake.

The influence of edelfosine on the internalization rates of SfbI wt- and SfbI ΔProD-coated beads is multifunctional and, although effecting different targets, might be the reason for the similar inhibitory effect since edelfosine not only inhibits the PLC and therefore inactivates phagocytic mechanisms, it also removes cholesterol from membranes (van Blitterswijk and Verheij, 2008; Hac-Wydro *et al*., 2011) and therefore might influence directly caveolae-dependent uptake processes. Components in the signal transduction pathway during phagocytotic processes play also a role in SfbI-mediated uptake mechanisms but only to a limited extent. This is not surprising since the signal cascades are intimately connected and thus might influence each other.

The results give evidence that signals of the classical phagocytic processes were bypassed by SfbI via the ProD region probably by distinct integrin-populations with consequences for the involvement of the downstream signals. However, work need to be done to clarify if the amount or the types of the involved integrins or other unknown aspects determine signal cascades and thus configure the entry process.

6.5 The function of the AroD region

When comparing the invasion mechanisms of two FnBPs, SfbI from GAS and GfbA from GGS, Rohde and coworkers observed completely different morphologies. While SfbI triggered aggregation and subsequent fusion of caveolae, GfbA expressing GGS were found inducing cytoskeleton rearrangements. GfbA proteins contain identical domain structure and show significant amino acid identity with SfbI proteins; the number of ProD repeats and FnBRs of SfbI and GfbA are highly similar, whereas the AroD regions differ considerably. It was therefore speculated that the AroD region somehow modulates the entry of streptococci.

To analyze the function of the AroD during the entry process more in detail Rohde and colleagues constructed a truncated GfbA derivate lacking the AroD region (GfbApro) and generated a chimeric SfbI construct in which the AroD region was replaced by the homologue region of GfbA (SfbIGaro). Those and the wildtype proteins were heterologous expressed on the surface of the naturally non invasive SGO and were found to induce bacterial entry in different ways. The AroD region of GfbA triggered cytoskeleton rearrangements, independent of the GfbA or SfbI background, whereas the truncated GfbA trigger a caveolae-dependent streptococcal uptake comparable to the SfbI-induced entry mechanism. Furthermore, intracellular trafficking was shown to depend on the bacterial entry mechanisms. Bacteria reside within caveosomes and prevent lysosomal fusion when the entry into host cells was based on the aggregation and subsequent fusion of caveolae which was found to be induced by SfbI and GfbApro. In contrast GfbA and SfbIGaro trigger bacterial uptake via large cytoskeleton rearrangements followed by trafficking intracellular along the classical endocytic pathway with final lysosomal fusion to form phagolysosomes finally degrading streptococci (Rohde *et al*., 2003; 2011).

It was found in the present study that SfbI and GfbApro which were shown to induce bacterial entry by co-opting caveolae induce remarkable integrin clustering and the uptake of gold-aggregates by large invaginations in the cell membrane. GfbA and SfbIGaro causing cytoskeleton rearrangements when heterologous expressed on SGO were unable to trigger detectable integrin-clustering or caveolae-aggregation, instead membrane alterations on the cellular surface were induced and only single or pair-wise gold-nanoparticles were found comparable to the results of SfbI lacking the ProD region, leading to the suggestion that the AroD region of GfbA contains *e.g*. structural information that prevent integrin-clustering and rather trigger cytoskeleton rearrangements.

The question remains how the AroD region realizes the modulatory effect(s) since no structural information about this domain and about the overall organization of FnBPs is available. The AroD region was shown to be hypervariable due to horizontal recombination

(Towers *et al*., 2003) and its effect is supposed to be due to conformational regulation of the SfbI protein and therefore of the SfbI domains. It can only be speculated that the AroD regions of distinct types, exhibiting different conformational structures, may interfere with the Fn-binding capacity of the Fn-binding domain preventing a “critical Fn-density” or affect the way Fn is bound with consequences for the interaction with other ECM molecules or with cell surface integrins (for further discussion see above). Also a conformational regulation-based effect on the ProD region influencing its attributes and thus the ProD triggered entry process is possible.

A potential support of the AroD on the SfbI-dependent internalization is further indicated by bead-host cell co-incubation assays. Four FnBRs and the AroD were sufficient to induce internalization of beads coated with recombinant SfbI ΔProD. However, the ProD region might support invasion characteristics since beads coated with recombinant SfbI lacking the FnBRs, thus, including the ProD and the AroD, or lacking the FnBRs and the AroD, thus, just consisting of the ProD, showed indeed reduced but still significant adherence (approximately 70 % or 50 %, respectively, compared to the SfbI wt) and invasive (approximately 30 % or 15 %, respectively, of adherent beads) capacities.

Moreover, in this study a correlation of the intracellular trafficking pathway and the uptake mechanisms was observed with consequences for intracellular surviving SGO heterologous expressing the distinct SfbI/GfbA constructs. The obtained results lead to the hypothesis that the AroD region modulates the early steps of the entry process by regulating the ProD capacity to fine-tuning integrin-clustering, which might lead to distinct invasion mechanisms resulting in a corresponding intracellular trafficking route and subsequently affecting the bacterial survival and hypothetical also the infection outcome.

Surface immobilization of the recombinant protein is a limiting factor of the bead-system since it permits translocation of SfbI into and a hypothetical effect within the host cell cytosol. Although slightly different intracellular behavior, i.e. (co-)localization with lysosomal compartments could be observed in the present study, this might preferential be due to the dependency of intracellular trafficking on the internalization process shown by studying the AroD region and not on further inhibitory effects of ProD on Arp2/3 complex within the cytosol. Cytosolic translocation of SfbI might be unique to GAS; from that reason also the SGO system might be limited for studying the intracellular effects triggered by SfbI which

could explain the general time-dependent reduction of intracellular SGO heterologous expressing distinct SfbI or GfbA proteins.

Nevertheless, the recombinant protein was successfully immobilized on the surface of latex beads; the co-incubation assays of host cells with recombinant protein coated beads provide an excellent tool to study the early events during entry processes into host cells and allow gaining insights into the SfbI-triggered internalization mechanisms excluding any side-effect of other bacterial factors.

Results of the present study give strong evidence that the ProD region play a prominent part in the invasion pattern of the SfbI-induced internalization processes of GAS by inducing clustering of a heterogenic integrin-population, i.e. by the DGE-motifs of the ProD repeats, and therefore preventing a phagocytic-like internalization of streptococci which demonstrates for the first time a functional role of the ProD region.

A correlation of bacterial survival and the invasion mechanisms could be shown since the intracellular trafficking route and thus the fate of intracellular bacteria depends at least to a certain extent on the entry process.

Our findings lead to the hypothesis that the structure of the streptococcal virulence factor SfbI, meaning the AroD region and/or the number of the ProD repeats and FnBRs, influences the outcome of a GAS infection by modulating the invasion into host cells and by a potential direct interaction of the ProD repeats with the cellular Arp2/3 complex, thus manipulating actin-dependent endosomal motility and avoiding lysosomal fusion.

Thus, SfbI is not only a highly multifunctional virulence factor it might also represent the first identified bacterial inhibitor of the Arp2/3 complex.

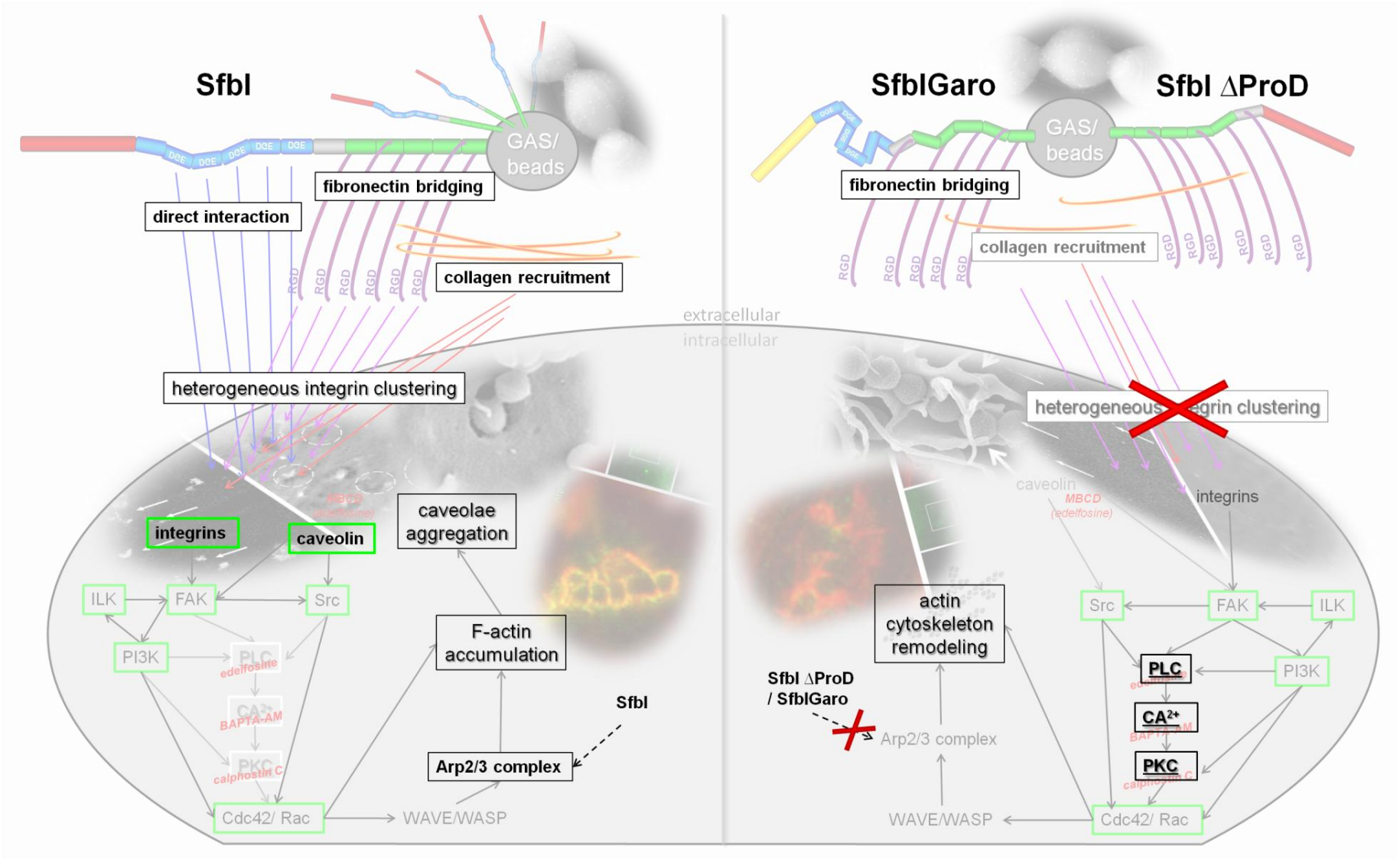

Figure 28: Schematic overview of the results obtained from the present study in combination with hypothesized specifications and with published data. Intracellular signals which were already shown to be involved in SfbI-mediated uptake were depicted in green boxes. Factors playing a role in the respective internalization processes mediated by SfbI (left side) or SfbIGaro and SfbIΔProD (right side) are accentuated and encircled.

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CURRICULUM VITAE

PERSONAL DETAILS

Name	Katja Branitzki-Heinemann
	Ahlumer Weg 4
	D-38300 Wolfenbüttel
Date of birth	27th of November 1984
Nationality	German

EDUCATION 2004 - 2013

10/2009 – 04/ 2013	PhD student in Infection Biology, Helmholtz Centre for Infection Research (HZI), Dep. Medical Microbiology, Germany. Topic: *Functional analysis of different streptococcal invasion mechanisms and consequences for intracellular survival*.
10/2007 – 09/2009	Master of Science (biology), Technical University Carolo-Wilhelmina, Braunschweig, Germany. Master thesis at the University of California, San Diego, School of Medicine, Division of Pediatric Pharmacology & Drug Discovery, USA. Topic: *The role of HIF-1α-associated signal transduction pathways in the antimicrobial activity of mast cells against Gram-positive infections.*
10/2004 – 07/ 2007	Bachelor of Science (biology), Technical University Carolo-Wilhelmina, Braunschweig, Germany. Bachelor thesis at the Helmholtz Centre for Infection Research (HZI), Dep. Medical Microbiology, Research Group Infection Immunology, Germany. Topic: *Mast cell responses following exposure to Staphylococcus aureus*.

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